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<p>(21) International Application Number: PCT/GB97/00955</p> <p>(22) International Filing Date: 4 April 1997 (04.04.97)</p> <p>(30) Priority Data:</p> <table> <tr> <td>9607289.7</td> <td>9 April 1996 (09.04.96)</td> <td>GB</td> </tr> <tr> <td>9608445.4</td> <td>24 April 1996 (24.04.96)</td> <td>GB</td> </tr> <tr> <td>9704905.0</td> <td>10 March 1997 (10.03.97)</td> <td>GB</td> </tr> </table> <p>(71) Applicant (for all designated States except US): BRITISH TECHNOLOGY GROUP LTD. [GB/GB]; 101 Newington Causeway, London SE1 8BU (GB).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): LATHE, Richard [GB/GB]; University of Edinburgh, West Mains Road, Edinburgh EH9 3JQ (GB). ROSE, Kenneth, Andrew [GB/GB]; University of Edinburgh, West Mains Road, Edinburgh EH9 3JQ (GB). SECKL, Jonathan, Robert [GB/GB]; Molecular Medicine Centre, Molecular Endocrinology, The University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU (GB). BEST, Ruth [GB/GB]; Molecular Medicine Centre, Molecular Endocrinology, The University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU (GB). YAU, Joyce, Lai, Wah [GB/GB]; Molecular Medicine Centre, Molecular Endocrinology, The University</p>		9607289.7	9 April 1996 (09.04.96)	GB	9608445.4	24 April 1996 (24.04.96)	GB	9704905.0	10 March 1997 (10.03.97)	GB	<p>of Edinburgh, Western General Hospital, Edinburgh EH4 2XU (GB). LECKIE, Caroline, McKenzie [GB/GB]; Molecular Medicine Centre, Molecular Endocrinology, The University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU (GB).</p> <p>(74) Agent: DOLAN, Anthony, Patrick; British Technology Group Ltd., Patents Dept., 101 Newington Causeway, London SE1 6BU (GB).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>	
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<p>(54) Title: USE OF 7 ALPHA-SUBSTITUTED STEROIDS TO TREAT NEUROPSYCHIATRIC, IMMUNE OR ENDOCRINE DISORDERS</p> <p>(57) Abstract</p> <p>Use is provided for a 7α-hydroxy or 7-oxo substituted 3β-hydroxy-steroid possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, or an analogue thereof substituted independently at one or both of the 7- and 3-positions with an ester or ether group, in the manufacture of a pharmaceutical composition for the therapy of neuropsychiatric, immune and/or endocrine disorders or for inducing cognitive enhancement. Uses for Cyp7b enzymes in producing such steroids is also provided together with various novel steroids and test kits and methods for diagnosing the disorders.</p>												

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USE OF 7 ALPHA-SUBSTITUTED STEROIDS TO TREAT NEUROPSYCHIATRIC, IMMUNE OR ENDOCRINE DISORDERS

The present invention relates to novel uses for 7 α -hydroxy-substituted steroids, to a process for preparing such steroids and to novel steroids so produced.

In particular the invention relates to the use of cytochromes of the cytochrome P450 family designated Cyp7b to effect 7 α -hydroxylation of certain 3 β -OH steroids so as to produce a 7 α -hydroxy-substituted steroids. Certain of the 7 α -hydroxy-substituted steroids so produced, as well the corresponding 7-oxo derivatives, are novel and form further aspects of the invention. The invention also relates to uses of these steroids, to uses of Cyp7b enzymes and to uses of novel macromolecular species, eg. antibodies and DNAs, 10 which are biologically related to the Cyp7b enzymes.

Cytochromes P450 are a diverse group of heme-containing mono-oxygenases (termed CYP's; see Nelson *et al.*, *DNA Cell Biol.* (1993) 12, 1-51) that catalyse a variety of oxidative conversions, notably of steroids but also of fatty acids and xenobiotics. While CYP's are most abundantly expressed in the testis, ovary, placenta, adrenal and liver, it is 15 becoming clear that the brain is a further site of CYP expression. Several CYP activities or mRNA's have been reported in the nervous system but these are predominantly of types metabolizing fatty acids and xenobiotics (subclasses CYP2C, 2D, 2E and 4). However, primary rat brain-derived glial cells have the capacity to synthesize pregnenolone and progesterone *in vitro*. Mellon and Deschepper, *Brain Res.* (1993), 629, 283-292(9) 20 provided molecular evidence for the presence, in brain, of key steroidogenic enzymes CYP11A1 (scc) and CYP11B1 (11 β) but failed to detect CYP17 (c17) or CYP11B2 (AS). Although CYP21A1 (c21) activity is reported to be present in brain, authentic CYP21A1 transcripts were not detected in this tissue.

Interest in steroid metabolism in brain has been fuelled by the finding that adrenal- 25 and brain-derived steroids (neurosteroids) can modulate cognitive function and synaptic plasticity. For instance, pregnenolone and steroids derived from it are reported to have memory enhancing effects in mice. However, the full spectrum of steroid metabolizing CYP's in brain and the biological roles of their metabolites *in vivo* has not been established.

Many aspects of brain function are modulated by steroids. Intracellular receptors 30 for glucocorticoids (cortisol, corticosterone) are particularly abundantly expressed in the

hippocampus (1), a brain region that plays a key role in specific aspects of memory formation, and which is an early and prominent target for dysfunction and damage in Alzheimer's disease (AD). While glucocorticoids regulate learning and memory, mood and neuroendocrine control, chronic glucocorticoid excess compromises neuronal activity, 5 synaptic plasticity and eventually survival, particularly in the hippocampus. These findings prompted the suggestion that glucocorticoid-mediated neurotoxicity might underpin some age-related brain disorders, including AD, in which plasma cortisol levels are markedly elevated (2).

Conversely, dehydroepiandrosterone (DHEA), the most abundant steroid product 10 of the human adrenal cortex, has been proposed to protect against disorders of the aging brain (3). Plasma levels of DHEA often show a striking age-associated decline which correlates with loss of cognitive function (4). In rodents, injection of DHEA or its sulfate into limbic structures improves post-training memory and enhances synaptic plasticity (5). DHEA and glucocorticoids thereby appear to exert inverse effects upon memory function 15 and synaptic plasticity, and DHEA has been advocated as an endogenous 'anti-glucocorticoid'. However, despite considerable circumstantial evidence to support this contention, there is no evidence for a direct interaction between DHEA and glucocorticoid signalling pathways in neurons.

Neurosteroidogenesis has been reported in isolated rat retina (8) and brain (9). In 20 addition to the production of pregnenolone and DHEA from cholesterol, a variety of novel steroids are made in brain extracts or cultured brain cells, including 20 α -dehydro pregnenolone, 7 α -hydroxy derivatives of pregnenolone and DHEA, progesterone, and both 3 α - and 3 β -hydroxy-5 α -pregnan-20-one (reviewed in Ref. 7). Androgens are also modified, particularly through the action of aromatase and a 5 α -reductase (reviewed in Ref. 25 10). However, the specific enzymes responsible for these and other transformations in the central nervous system have not been well characterized.

As referred to above, several Cyps are present in the central nervous system (11-22). Activities or mRNAs corresponding to key steroidogenic enzymes (23-25), in addition to 30 Cyp19 (aromatase) have been detected. Furthermore, mRNAs encoding the non-Cyp hydroxysteroid dehydrogenases (HSD) 3 α -HSD, 3 β -HSD and 11 β -HSD have been reported in the central nervous system (25, 27-29).

To investigate regulation of brain function, studies reported in copending International Patent Application No PCT/GB95/02465, published as WO 96/12810, and in Stapleton *et al* (J. Biol. Chem. 270, 29739 - 1995, December, 15 1995), focused on the hippocampus, a brain region important in learning and memory. A copy of the specification 5 of International Patent Application No PCT/GB95/02465 has been filed with the priority documents filed in respect of this specification.

That copending application, PCT/GB95/02465, describes and claims novel cytochrome P450 proteins designated Hct-1. These Hct-1 proteins have now been named as Cyp7b by the Committee on Standardized Cytochrome P450 Nomenclature and the 10 name Cyp7b will be used in this application.

The Cyp7b enzyme shares 39% sequence identity to hepatic cholesterol 7 α -hydroxylase (Cyp7a) and lesser but significant homology with other steroidogenic Cyps. The postulated steroidogenic domain (30,31), found in many of these enzymes, is present 15 in both Cyp7a and Cyp7b. Cyp7b mRNA is predominantly expressed in rodent brain, particularly in the hippocampus, unlike Cyp7a, which is liver-specific (31-33 and EP0648840 A2).

The present inventors have now investigated the substrate specificity of Cyp7b and found that Cyp7b catalyses the introduction of a hydroxyl group at the 7 α position in steroid substrates, particularly 3 β -hydroxy steroids. Cytochromes Cyp7b are thus steroid 20 hydroxylase enzymes having 7 α -specificity. The ability to produce 7 α -hydroxylated steroids is of major commercial importance, because such steroids are of particular use in the manufacture of pharmaceuticals (either as drugs *per se* or as intermediates), and in the manufacture of test kits and assays for pathological conditions associated with the presence of abnormal levels of endogenous enzyme, substrate or product.

25 The abbreviation "DHEA" will be used herein to designate dehydroepiandrosterone, thus 7 α -hydroxy-DHEA designates 7 α -hydroxydehydroepi-androsterone

The present inventors have identified substrate/product pairs associated with Cyp7b, particularly DHEA/7 α -hydroxy-DHEA (7-HD), pregnenolone/7 α -hydroxy-pregnenolone (7-HP) and β -estradiol/7 α -hydroxy- β -estradiol (7-HE). They have also determined that 30 DHEA concentration in brain tissue declines with age, whereas the concentrations of other brain steroids do not, and determined that the ageing process may be associated with

deficits in certain steroids and also with deficits in the concentration of Cyp7b itself. It is also believed that one of the products produced by Cyp7b mediated reactions, namely 7 α -hydroxy dehydroepiandrosterone, plays an important role in the operation of the immune system. Because 7 α -hydroxy-DHEA is believed to be made substantially only in
5 the brain, the inventors hypothesize that senescence may be due to a deficit in brain-produced 7 α -hydroxy-DHEA as well as in other steroids found in the brain such as DHEA, pregnenolone and 7 α -hydroxy-pregnenolone.

The present inventors have now further determined that one of the specific properties of the 7 α -hydroxy-substituted steroids, and potentially their 7-oxo substituted steroid derivatives, provided by the present invention is that of glucocorticoid and/or mineralocorticoid antagonism, whether at receptor level or otherwise. This is particularly demonstrated by the Example 5 below with respect to 7 α -hydroxy-DHEA but is more generally applicable. Thus this activity not only gives further uses for the novel steroids of the invention but provides first and second medical uses for known 7 α -hydroxy or 7-oxo
10 steroids made available by the present process as glucocorticoid and/or mineralocorticoid
15 antagonists and preferably in antagonism specific to neuronal tissue such as in the CNS.

Thus, having regard to this activity and their involvement in endogenous metabolic pathways, particularly in the brain, the 7 α -hydroxy substituted 3 β -hydroxy-steroids provided by use of the Cyp7b enzyme activity, including novel compounds provided by the
20 invention, and their 7-oxo derivatives, have utility in the therapy of neuropsychiatric, immune and endocrine disorders, particularly but not exclusively steroid associated disorders.

Use of these 7 α -hydroxy or 7-oxo substituted 3 β -hydroxy-steroids, preferably possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, or
25 derivatives thereof substituted independently at one or both of the 7- and 3- positions with an ester or ether group, in treating these disorders and for manufacturing medicaments for such treatment is provided in a first aspect of the present invention. Particularly preferred derivatives are those wherein one or both of the ester and or ether group is metabolisable
in vivo to produce the corresponding hydroxy compound.

30 Preferred derivatives include those wherein the steroid has a 3 β -substituent-OR₁ and/or a 7 α -substituent -OR₂ where -OR₁ and -OR₂ each independently represents a free

hydroxy, ester or ether group,

wherein each of R_1 and R_2 are independently selected from the group consisting of hydrogen, substituted or unsubstituted C_{1-6} alkyl groups, groups R_5CO- , wherein R_5 may be selected from substituted or unsubstituted C_{1-6} alkyl groups, and groups of the formula $-OP(OH)_3$, wherein any substituents are selected from OH, halogen (F, Cl, Br, I) amino, C_{1-6} alkylamino, C_{1-6} dialkylamino, COOH or $COOR_4$ wherein R_4 represents a C_{1-6} alkyl group; and wherein the compounds may be in free form or in the form of acid addition salts with pharmacologically acceptable anions.

The particular disorders for which this utility is provided include

- 10 (a) deficits of cognition in aging
- (b) Alzheimer's disease
- (c) deficits of immune system in aging
- (d) deficits of immune function in HIV infection
- (e) glucocorticoid or mineralocorticoid excess
- 15 (f) diabetes
- (g) depression
- (h) osteoporosis and hypercalcemia
- (i) hyperglycemia and hyperlipidemia
- (j) muscle atrophy
- 20 (k) arterosclerosis
- (l) steroid diabetes

Further, these 7α -hydroxy steroids, their esters, ethers and 7-oxo derivatives may be used to induce cognitive enhancement in a normal individual.

Preferred steroids for such use have the carbon skeleton of androsterone, pregnenolone or estradiol and particularly preferred examples are 7α -hydroxy-DHEA and 7α -hydroxypregnenolone. Accordingly the present invention further provides the use of novel compounds of Formula Ia and Ib shown below in the applications indicated above.

Particularly preferred uses for the antagonistic properties of these 7-substituted steroids include treatment of disorders falling within category (e) above or where reversal 30 of the effects of such corticoids, regardless of excess, is required.

A second aspect of the present invention provides pharmaceutical compositions implementing such use. The compositions in which the novel steroids and known steroids of the invention will be used will readily occur to those skilled in the art, generally comprising the steroid active in association with a pharmaceutically acceptable carrier or diluent, with formulations for example being suitable for inhalation or for gastrointestinal (e.g. oral), parenteral, topical, transdermal or transmucosal administration.

As an alternative to administering the compounds of the invention *per se*, a third aspect of the invention provides the possibility of using the gene sequences of the Cyp7b genes in gene therapy in order to compensate for a deficiency in Cyp7b enzyme. In such 10 therapies, constructs comprising Cyp7b coding sequences can be packaged in conventional delivery systems, such as adenoviruses, vaccinia viruses, herpes viruses and liposomes and administered via a route which results in preferential targeting of a selected tissue, especially the brain. The invention further provides the possibility of using the gene 15 sequences of the Cyp7b genes in gene therapy in order to achieve the endogenous expression of Cyp7b sequences for other purposes, e.g. in order to promote immunogenic processes. Thus for example, a vector such as a suitably modified vaccinia virus (or variant thereof) may be co-administered with a vaccine formulation so that the expressed Cyp7b sequences augment the immunogenic properties of the vaccine.

It will be realised that in the event of Cyp7b related disorders other than those 20 involving its depletion it may be desirable to use vectors containing antisense sequences to Cyp7b effective such as to inhibit Cyp7b expression.

Macromolecules related immunologically to Cyp7b enzymes form fourth and fifth aspects of the invention and in this regard antibodies, particularly monoclonal antibodies which are capable of selectively binding Cyp7b, have utility in the diagnosis of disorders 25 (a) to (I) referred to above. Anti-Cyp7b antibodies (including monoclonal antibodies) as well as binding molecules comprising antibody fragments may be produced by known methods and used in test kits for assays for Cyp7b enzymes.

According to a sixth aspect of the invention, there is provided a process of producing a 7 α -hydroxy-substituted steroid which comprises subjecting a corresponding 30 steroid substrate having no hydroxyl substituent in the 7-position to hydroxylation in the presence of a Cyp7b steroid hydroxylase enzyme.

The Cyp7b steroid hydroxylase enzyme used in the process of the invention is preferably a Cyp7b enzyme described and claimed in the above-mentioned International Patent Application No PCT/GB95/02465 (and referred to therein as Hct-1). Such enzymes include (a) ones having the precise amino acid sequences described for mouse, rat and 5 human Cyp7b, (b) homologous enzymes from other species and (c) enzymes having amino acid sequences which differ from the sequences of enzymes included in definitions (a) and (b), but in which the capacity to catalyse the introduction of a 7α -hydroxyl group is not eliminated.

10 The amino acid sequence of suitable Cyp7b steroid hydroxylase enzymes may be defined in terms of the DNA coding sequences disclosed in International Patent Application No PCT/GB95/02465. Thus the Cyp7b steroid hydroxylase enzyme may have a sequence encoded by DNA coding sequences of Cyp7b enzymes selected from

- (a) Coding sequences of DNA molecules comprising the coding sequence for rat Cyp7b set forth in SEQ Id No: 1,
- 15 (b) Coding sequences of DNA molecules comprising the coding sequence for mouse Cyp7b set forth in SEQ Id No: 2,
- (c) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (a) or (b) under standard hybridization conditions defined as 2 x SSC at 65°C.
- 20 (d) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (a), (b) or (c) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.

The sequences (a) and (b) above represent rat and mouse Hct-1 gene sequence. 25 Homologous sequences from other vertebrate species, especially mammalian species (including man) fall within the class of DNA molecules represented by (c) or (d).

Thus for human Cyp7b, the steroid hydroxylase enzyme may comprise a sequence encoded by

(e) DNA coding sequences selected from the following:

(i) the sequence designated "exon 3" in SEQ Id No 3,
5 (ii) the sequence designated "exon 4" in SEQ Id No 3, and

(f) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (e) under standard hybridization conditions defined as 2 x SSC at 65°C.

10 (g) Cyp7b steroid hydroxylase encoding DNA molecules capable of hybridizing with the DNA molecule defined in (e) or (f) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.

15 (h) Cyp7b steroid hydroxylase-encoding DNA molecules comprising contiguous pairs of sequences selected from
 (i) the sequence designated "exon 3" in SEQ Id No 3,
 (ii) the sequence designated "exon 4" in SEQ Id No 3, and

15 (i) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (h) under standard hybridization conditions defined as 2 x SSC at 65°C.

20 (j) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (h) or (i) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.

- (k) Coding sequences of DNA molecules comprising a contiguous coding sequence consisting of the sequences "exon 3" and "exon 4" in SEQ ID No 3, and
- 5 (l) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (k) under standard hybridization conditions defined as 2 x SSC at 65°C.
- 10 (m) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (k) or (l) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.

It will be appreciated that the DNA sequences referred to may consist of or be derived from genomic DNA, but typically would consist of or be derived from cDNA. Such sequences could be obtained by probing an appropriate library (cDNA or genomic) using hybridisation probes based upon the sequences provided according to the invention 15 of International patent application No PCT/GB95/02465, or they could be prepared by chemical synthesis or by ligation of sub-sequences.

In the above definitions, Cyp7b steroid hydroxylases have been defined in terms of DNA sequence information. The Cyp7b steroid hydroxylase enzyme used in accordance with the process of the invention may alternatively or additionally be defined by reference 20 to amino acid sequence information, e.g. the amino acid sequences contained in SEQ ID NO. 4, SEQ ID NO. 5 or SEQ ID NO. 6.

Thus the Cyp7b steroid hydroxylase enzyme used in accordance with the process of the invention may have sequences matching one of said sequences exactly, or alternatively, the enzymes used may have sequences which differ from the aforementioned 25 sequences, provided that the capacity to catalyse the introduction of a 7 α -hydroxyl group is not eliminated.

Thus, for example, mutant enzymes may be produced by known methods, for example site-directed mutagenesis or other PCR-based procedures, and the expression

products tested for their capacity to catalyse the introduction of a 7α -hydroxyl group in selected substrates in accordance with the procedures described herein.

Having regard to the degree of homology between the rat, mouse and human enzymes and known data relating to species divergence of hydroxylase enzymes, it is 5 preferred that by comparison with the DNA sequences of SEQ ID NO. 1, SEQ ID NO. 2 and SEQ ID NO. 3, the mutant enzymes should be encoded by sequences having at least 50% homology, more preferably at least 60% homology and most preferably at least 70% homology with said sequences over a length of 50 contiguous nucleotides.

Preferably the mutant enzymes are encoded by sequences having at least 60% 10 homology with the entire coding sequence, more preferably at least 70%.

Alternatively, by comparison with the amino acid sequences of SEQ ID NO. 4, SEQ ID NO. 5 and SEQ ID NO. 6, it is preferred that mutant enzymes should have at least 50% homology, more preferably at least 60% homology and most preferably at least 70% homology with said sequences over a length of 30 contiguous amino acids. Preferably 15 the mutant enzymes have at least 60% homology and more preferably 70% homology or more with the entire amino acid sequence in each case.

It is however preferred that such mutant enzymes do not differ too drastically from the aforementioned sequences and in this regard, where amino acid substitutions are made, that the substituted amino acids are preferably so-called "synonymous" or "conservative" 20 substitutions. i.e. hydrophilic, hydrophobic, basic and acidic amino acids should preferably be substituted by amino acids in the same class (see US 5380712).

More specifically, it is preferred that the mutant enzymes differ from the precise sequences of those described herein by not more than 20, preferably not more than 10 and most preferably not more than 5 amino acid substitutions, insertions or deletions.

25 The Cyp7b enzymes described herein may be used in toxicological and drug evaluation studies and such uses form further aspects of the invention. In a particularly preferred embodiment of this aspect of the invention, a cell line capable of expressing a Cyp7b enzyme is used as a basis of an assay for one or more Cyp7b substrates. Such cell lines have utility in toxicological and drug evaluation studies. Most preferably the cell line 30 comprises a prokaryotic or eucaryotic cell line which has been transformed so as artificially to express a Cyp7b enzyme. Examples include bacteria, yeast and mammalian cells. Also

included are transgenic animals, at least one tissue of which (especially a non-brain tissue) expresses Cyp7b enzyme. Such transgenic animals may be produced by known methods for introducing foreign coding sequences into somatic or germ line cells.

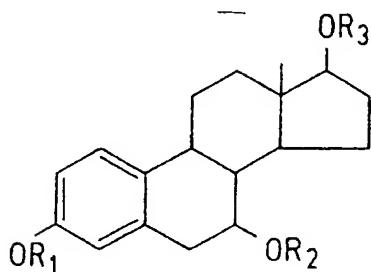
The substrates used in the method of the invention are characterised by possessing 5 a 3 β -hydroxyl group and further by preferably possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, with the proviso that where the substrate has the carbon skeleton of cholesterol, the substrate has a hydroxyl group in the 25, 26 or 27-position, preferably the 25-position.

Examples of such substrates include 25-hydroxycholesterol, dehydroepi-10 androsterone, pregnenolone and estradiol, in which case the steroids produced will be 7 α -hydroxy-25-hydroxycholesterol, 7 α -hydroxydehydroepiandrosterone, 7 α -hydroxy pregnenolone and 7 α -hydroxyestradiol (i.e. estra 1,3,5(10)-triene-3,7 α ,17 β -triol) respectively.

The 7 α -hydroxylated steroid produced according to the invention may be oxidised 15 by known enzymatic or non-enzymatic procedures to produce 7-oxo substituted steroids and this further process step forms a further aspect of the invention.

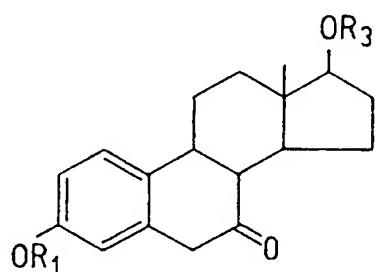
Certain 7 α -hydroxy-substituted steroids produced according to the invention and certain corresponding 7-oxo derivatives are novel and provide a further aspect of the invention. Thus the present invention further provides novel 3 β -hydroxy steroids 20 characterised in that they have a 7 α -hydroxy or 7-oxo substituent. Preferred novel steroids have the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, with the proviso that where the skeleton is that of cholesterol, the 25, 26 or 27 position is hydroxylated, most preferably the 25 position.

Particular novel steroids are of the formula



25

Ia



Ib

wherein OR₁, OR₂ and OR₃ each independently represents a free hydroxy group, an ether group or an esterified hydroxy group.

In the case where OR₁, OR₂ and OR₃ each independently represents an ether group, each of R₁, R₂ and R₃ may be selected from substituted or unsubstituted C₁₋₆ alkyl groups, any such substituents being selected from OH, halogen (F, Cl, Br, I) amino, C₁₋₆ alkylamino, C₁₋₆ dialkylamino, COOH or COOR₄ wherein R₄ represents a C₁₋₆ alkyl group which may be unsubstituted or substituted by one of the substituents referred to above.

In the case where OR₁, OR₂ and OR₃ each independently represents an esterified hydroxy group, each of R₁, R₂ and R₃ may have the formula R₅CO-, wherein R₅ may be selected from substituted or unsubstituted C₁₋₆ alkyl groups, any such substituents being selected from OH, halogen (F, Cl, Br, I) amino, C₁₋₆ alkylamino, C₁₋₆ dialkylamino, COOH or COOR₄ wherein R₄ represents a C₁₋₆ alkyl group; and groups of the formula -OP(OH)₃. Where compounds of Formula Ia or Ib include substituents such as carboxyl groups, phosphate groups, or substituted or unsubstituted amino groups, the compounds may be in free form or in the form of acid addition salts with pharmacologically acceptable anions (such as, for example, phosphate or halide ions) or cations (such as, for example, alkaline metal cations). Thus, where OR₁, OR₂ or OR₃ represents hemesuccinate HOOC(CH₂)₂CO, the resulting hemesuccinate may be in the form of, for example, an Na or K salt.

It will be realised that the present invention provides for 7 α -hydroxylated and 7-oxo steroids as described above but which are further substituted at other positions directly on the steroid skeleton.

7 α -Hydroxyestradiol and 7-oxoestradiol are specific examples of compounds of Formula Ia and Ib.

The invention will now be described in more detail with particular reference to the following Figures and Examples.

Description of Figures

Figure 1 illustrates an autoradiogram of a TLC plate used in an experiment to assess the ability of various cell extracts to hydroxylate DHEA.

Figure 2 depicts the ability of various tissues to release radioactivity from 7-³H-pregnenolone.

Figure 3 illustrates the principal steroid interconversions mediated by Cyp7b.

Figure 4 is a histogram plotting fold induction of luciferase expression with 5 concentration of various steroids as described in Example 5.

Figure 5 illustrates the attenuation of Cyp7b gene expression in Alzheimer's as described in Example 5.

Figure 6 shows mass spectrometer plots of 7 α -hydroxy-DHEA produced by the present process and a reference sample thereof.

10 **EXAMPLE 1 - Identification of substrate specificity of Mu Cyp7b**

A. **Preparation of vaccinia expression construct**

To identify the reaction catalysed by Cyp7b a cDNA encoding the mouse enzyme, reported by Lathe, Rose and Stapleton (PCT/GB95/02465) and by Stapleton et al. (J. Biol. Chem. 270, 29739-1995, December 15 1995), was modified to introduce a translation 15 initiation consensus sequence at the 5' end of the Cyp7b open reading frame as described therein. The modified cDNA was introduced into the genome of vaccinia virus by recombinational exchange according to standard procedures (see, for instance, Gonzalez et al., Meth. Enzymol. 206, 85-92, 1991 and references therein) as described in Lathe et al.

20 **B. Production of Cyp7b enzyme extracts.**

HeLa cells were grown to semi confluence (10^6 cells per 5 cm dish; 5 ml medium) and infected with recombinant (VV-Cyp7b) and control (VV Copenhagen strain) vaccinia viruses at 0.1 pfu per cell; 16 hours later infected cells were washed and taken up into W (Waxman) buffer (0.1 M KP04, 1 mM EDTA, 20% glycerol pH 7.5; 500 μ l per plate) and 25 recentrifuged (5 min., 1000 rpm).

For whole cell extracts cells were resuspended into 1/100 volume (50 μ l per plate) of W buffer and stored frozen at -70°C. For microsome preparation (Waxman, Biochem. J. 260, 81-85, 1989) cells were resuspended in 1/10 original volume of W buffer (500 μ l per plate); sonicated 6 x 5 seconds on ice, and unbroken cells were removed by 30 centrifugation (10 min., 4°C, 3000 rpm).

The microsomal fraction was prepared from the supernatant by centrifugation

(100,000 g, 45 min., 4°C, Beckman SW50.1 rotor) and resuspended using a Potter homogeniser in 1/50 original volume of W buffer (100 µl per plate) before storage at -70°C.

Control extracts were prepared from liver and brain from male rat by homogenising 5 fresh tissue in W buffer (2.5 ml/g), clarifying briefly by centrifugation (4000 rpm, 5 min, 4°C); the supernatant was stored at -70°C.

C. Substrate identification by thin-layer chromatography.

10 ^{14}C or ^3H -labelled steroids were purchased from DuPont-NEN (^{14}C -labelled molecules: specific activities 45-60 mCi/mmol.; ^3H : specific activities 70-100 mCi/mmol). 1 nMol aliquots of labelled substrate were dried down, microsomes or cell and tissue extracts were added (25 to 50 µl), and diluted to a volume of 175 µl with W buffer.

15 Reaction was started by the addition of 25 µl of 8 mM NADPH. After incubation at 37°C for 15 minutes the reaction was shaken with 500 µl of ethyl acetate (BDH). The organic phase was removed, dried down, and suspended into 10 µl ethyl acetate. Aliquots (5 x 2 µl) were applied to thin layer chromatography (TLC) sheets (Merck) and developed in ethyl acetate/n-hexane/acetic acid 16:8:1 (solvent system N of Waxman, Meth. Enzymol. 206, 462-476, 1991). After drying, chromatograms ^{14}C were visualised by exposure to X-ray film. ^3H -labelled chromatograms were treated with EN ^3H HANCE™ (DuPont-NEN) 20 spray prior to exposure.

D. Results

Figure 1 is an autoradiogram of a TLC plate run in solvent system N; substrate was ^3H -DHEA and samples were extracted with ethyl acetate and dried prior to application to the TLC plate (origin at bottom of figure). Extracts were 1, Microsomes from HeLa cells 25 infected with control vaccinia virus (negative control); 2, Microsomes from HeLa cells infected with VVCyp7b; 3, Duplicate preparation of microsomes from HeLa cells infected with VVCyp7b; 4, Rat brain homogenate.

As can be seen from Figure 1, microsomes from cells infected with recombinant vaccinia expressing Cyp7b converted ^{14}C -dehydroepiandrosterone (DHEA) to a lower 30 mobility form most consistent with hydroxylation. Brain extracts yielded a product of indistinguishable mobility, consistent with our earlier demonstration that Cyp7b is

expressed in brain. From the relative mobility of the product we surmised that Cyp7b could be hydroxylating DHEA at the 7 position. Progesterone, corticosterone, cortisol and testosterone were at best inefficiently metabolised, if at all. However, pregnenolone and estradiol were both converted by the enzymes, as was 25-hydroxy cholesterol. All these substrates are distinguished by a 3β hydroxy group.

EXAMPLE 2 - Identification of the position of the modification by ^3H -release.

To Identify the position of the modification, ^3H -pregnenolone (NEN) was employed in which the ^3H substitution was predominantly at the 7 position on the steroid backbone. Microsomal extracts were incubated with ^3H -pregnenolone under the same conditions as used earlier. Following reaction, labelled steroids were extracted with ethyl acetate (2 x 1 ml), and discarded; release of ^3H into the aqueous phase was monitored by liquid scintillation counting.

Referring to Figure 2, 7- ^3H -pregnenolone was incubated with extracts and assayed for release of radioactivity into the aqueous phase following extraction with ethyl acetate. Extracts were 1, Microsomes from Hela cells infected with control vaccinia virus (negative control); 2, Microsomes from Hela cells infected with VVCyp7b; 3, Duplicate preparation of microsomes from Hela cells infected with VVCyp7b; 4, Rat brain homogenate; 5, Rat liver homogenate.

As seen in Figure 2 microsomes from cells infected with recombinant vaccinia expressing Cyp7b efficiently released ^3H into the aqueous phase. Brain also performed this reaction but not liver. Release of ^3H from the 7 position of pregnenolone demonstrates that Cyp7b hydroxylates pregnenolone at the 7-position to generate 7-hydroxy pregnenolone (7HP); it may be concluded that Cyp7b also hydroxylates DHEA (to generate 7-hydroxy DHEA [7HD]) and estradiol to generate 7-hydroxy estradiol [7HE].

25

EXAMPLE 3 - Stereochemistry of the Cyp7b hydroxylation.

Steroids hydroxylated at a variety of positions (egs. 2, 6, 7, 15, 16) differ in their mobility on TLC depending on whether the modification is in the α - or the β -position (Waxman, Meth. Enzymol. 206, 462-476, 1991). Purified 7α -hydroxy DHEA was obtained (kind gift of Dr. H.A. Lardy, Enzyme Institute, University of Wisconsin), mixed with the product of Cyp7b action on DHEA, and subjected to TLC. The product

comigrated with 7 α - hydroxy-DHEA, demonstrating that Cyp7b is a 7 α hydroxylase.

EXAMPLE 4 - Activity of enzyme in 7 α -hydroxylation of pregnenolone and DHEA

To examine the catalytic activity of the enzyme Cyp7b mRNAs were expressed in mammalian cell lines. Cell extracts showed substantial NADPH-dependent conversion of 5 DHEA (Km 13.3 μ M; Vmax 288pmol/min/mg) and pregnenolone (Km 3.6 μ M; Vmax 34 pmol/min/mg) to slower migrating forms on thin layer chromatography. Products of identical mobility were generated by rat brain extracts. The expressed enzyme was less active against 25-hydroxycholesterol, 17 β -estradiol and 5 α -androstane-3 β , 17 β -diol, with low to undetectable activity against progesterone, corticosterone and testosterone. When 10 [3 H-7 α] pregnenolone was incubated with Cyp7b extracts the extent of release of radioactivity into the medium suggested that hydroxylation was preferentially at the 7 α -position. In gas chromatography and mass spectrometry of the modified steroid arising from incubation of DHEA with Cyp7b extracts, the retention time and fragmentation patterns were identical to those obtained with authentic 7 α -hydroxy DHEA (7HD); the 15 reaction product also co-migrating with 7HD on TLC.

Mass spectrometry: A 10x scaled up reaction was employed using 95% unlabelled DHEA (Sigma) and 5% [14 C]-DHEA (final specific activity 2.25-3mCi/mmol) and reaction time was extended to 1 hour. Product was purified by TLC, excised and extracted with ethyl acetate before drying down. The dried residue and authentic 7HD (50mg) were 20 converted to their methoxime -trimethylsilyl (MO-TMS) derivatives. Analysis of these products was performed using a Trio 100 mass spectrometer operating in electron impact (EI) mode, linked to a HP5890 gas chromatograph fitted with a HP-1 cross-linked methyl siloxane column (25m, i.d. 0.25mm, 0.17 mm film) under the following conditions: 25 electron energy 70eV, source temperature 200°C, interface temperature 280°C, oven temperature 50°C increasing at 30°C per minute to 200°C, and then 10°C per minute to 300°C, injection temperature 280°C.

EXAMPLE 5 - Cis-trans co-transfection assay; demonstration of antagonism.

Chinese hamster ovary (CHO) cells were maintained and transfected in Dulbecco's 30 modification of Eagle's medium (DMEM) supplemented with 15% foetal bovine serum, 100IU/ml penicillin, 100 μ g/ml streptomycin and 200mM L-glutamine (all Gibco BRL,

Paisley, UK).

24 hours prior to transfection CHO cells were plated at a density of 3×10^5 /60 mm dish (Costar UK). Cells were transfected by the calcium phosphate method. Briefly, 5 μ g of MMTV-LUC and 1 μ g of pRShGR or 5 μ g of pSV2 as a control for transfection efficiency were made up to a total of 10 μ g/plate of DNA with pGEM3. 30 μ l of 2.5M CaCl₂ was diluted ten-fold with sterile water and 300 μ l was added to the DNA. Next 300 μ l of 2 x Hepes buffered saline (280 mM NaCl, 10mM Kcl, 1.5mM Na₂HPO₄.2H₂O), 50mM Hepes, 12mM dextrose, pH 7.05) was added slowly with swirling to the DNA/CaCl₂ mixture. This solution was left for 30 minutes in order for a fine precipitate to form and 10 600 μ l was added dropwise to each plate. After 24 hours the medium was removed and the cells were washed in serum free medium and culture for a further 24 hours in medium containing 10% charcoal-stripped serum together with the appropriate concentrations of DHEA/7 α -hydroxy-DHEA.

15 Six hours after the addition of DHEA/7 α -hydroxyDHEA either B or Dex was added to each plate. The following day the cells were washed in PBS, lysed with 0.3ml of lysis buffer (25mM Tris-phosphate pH 7.8, 2mM DTT, 1% Triton X-100 and 10% glycerol), scraped, centrifuged and the supernatant assayed in duplicate in a Berthold luminometer in a total volume of 250 μ l, comprising 40 μ l of cell extract, 5 μ l of 30mM ATP, 100 μ l of assay buffer (20mM tricine, 1.07nM (MgCO₃)₄.Mg(OH)₂.%H₂O, 2.67mM MgSO₄, 20 0.1mM EDTA, 33.3mM DTT, 0.2mg/ml coenzyme A) and 105 μ l luciferin (Promega UK) injected to initiate the reaction. Light emission was measured over 10 seconds and relative light units/microgram of protein was calculated.

25 Results are shown in Figure 4 wherein the fold induction of luciferase is illustrated by histogram for control, additions of DHEA, 7 α -hydroxy-DHEA (7HD) alone and these additions in presence of an GR activating concentration of corticosterone. This result shows that 7HD, but not DHEA, acts as an antagonist of corticosterone effect in activating the GR-mediated transcription.

EXAMPLE 6 - Cyp7b expression in Alzheimers neurons

30 Cryostat brain sections (10 μ m) from control and Alzheimer's hippocampus were cut, thaw mounted onto gelatine-subbed poly-L-lysine coated slides and stored at -80°C.

For *in-situ* hybridization studies, brain sections were post-fixed in 4%

paraformaldehyde by acetylation (0.25% acetic anhydride in 0.1M triethanolamine, pH 8.0) for 10 minutes, rinsed in phosphate buffered saline, dehydrated through graded alcohols and air dried. Hybridization was carried out using 200 μ l of [35 S]-UTP-labelled cRNA antisense probe (10×10^6 dpm/ml in hybridization buffer) synthesized *in vitro* from a 500 5 bp XbaI/PstI fragment of the human Cyp7b pMMC1 clone linearised with XbaI and transcribed with T3 RNA for sense probes. Sections were prehybridized with 20 μ l of prehybridization buffer (as hybridization buffer but omitting the dextran sulphate) per slide at 50°C for 3 hours.

Following hybridization with probe at 50°C overnight sections were treated with 10 RNase A (30 μ g/ml, 45 minutes at 37°C) and washed to a final stringency of 0.1 x SSC at 60°C. Slides were dehydrated, dipped in photographic emulsion (NTB-2, Kodak) and exposed at 4°C for 5 weeks before being developed and counterstained with 1% pyronin. The density of silver grains was assessed over individual hippocampal neurons by 15 computer-assisted grain counting using an image analysis system (Seescan plc, Cambridge, UK), with the analysis carried out blind (sections were cut and coded by a separate individual). For each slide, one hippocampal section represents each subject. 6-10 neurons/subregion were assessed and background, counted over areas of white matter, was subtracted. Data were assessed by ANOVA followed by Scheffe post hoc test. Significance was set at p<0.05. Values are means \pm S.E.M.

20 Figure 5 is a histogram showing Cyp7b expression as indicated by grain count per neuron in the dentate gyrus. CA1 and CA3 subfields of Alzheimer's disease samples as compared to the age matched control brains.

CONCLUSIONS

It can be concluded that Cyp7b, and cognate enzymes from rat, human and other 25 mammalian species, are 7 α -hydroxylases specific for steroid substrates with a 3 β hydroxy group. While activities for 7-hydroxylating DHEA, pregnenolone and cholesterol have been recorded previously in a variety of crude tissue homogenates (eg. Akwa et al., Biochem. J. 288, 959-964, 1992) no characterisation of the enzyme responsible was performed previously and no activity on estradiol was recorded. Recombinant organisms expressing 30 Cyp7b thus provide a route to the large scale manufacture of 7HP, 7HD, and 7HE, principally but not exclusively for therapeutic use or for the production of further steroid derivatives such as 7-oxo molecules.

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CLAIMS

1. The use of a 7α -hydroxy or 7-oxo substituted 3β -hydroxy-steroid, or a derivative thereof substituted independently at one or both of the 7- and 3- positions with an ester or ether group, in the manufacture of a pharmaceutical composition for the therapy of neuropsychiatric, immune and/or endocrine disorders or for inducing cognitive enhancement.
5
2. The use according to Claim 1 wherein said disorders are selected from
 - (a) deficits of cognition in aging
 - 10 (b) Alzheimer's disease
 - (c) deficits of immune system in aging
 - (d) deficits of immune function in HIV infection
 - (e) glucocorticoid or mineralocorticoid excess
 - (f) diabetes
 - 15 (g) depression
 - (h) osteoporosis and hypercalcemia
 - (I) hyperglycemia and hyperlipidemia
 - (j) muscle atrophy
 - (k) arterosclerosis
 - 20 (l) steroid diabetes
3. The use as claimed in claim 1 or claim 2 wherein the steroid has a 3β -substituent -OR₁ and/or a 7α -substituent -OR₂ where -OR₁ and -OR₂ each independently represents a free hydroxy, ester or ether group,
25 wherein each of R₁ and R₂ are independently selected from the group consisting of hydrogen, substituted or unsubstituted C₁₋₆ alkyl groups, groups R₅CO-, wherein R₅ may be selected from substituted or unsubstituted C₁₋₆ alkyl groups, and groups of the formula -OP(OH)₃, wherein any substituents are selected from OH, halogen (F, Cl, Br, I) amino, C₁₋₆ alkylamino, C₁₋₆ dialkylamino, COOH or COOR₄ wherein R₄ represents a

C_{1-6} alkyl group; and wherein the compounds may be in free form or in the form of acid addition salts with pharmacologically acceptable anions.

4. The use as claimed in any one of claims 1 to 3 characterised in that the steroid is one possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol,
5. 5. The use of a Cyp7b steroid hydroxylase enzyme in the manufacture of a test kit for use in the diagnosis of neuropsychiatric, immune and endocrine disorders.
6. The use according to Claim 5 wherein said disorders are selected from
 - (a) deficits of cognition in aging
 - (b) Alzheimer's disease
 - 10 (c) deficits of immune system in aging
 - (d) deficits of immune function in HIV infection
 - (e) glucocorticoid or mineralocorticoid excess
 - (f) diabetes
 - (g) depression
 - 15 (h) osteoporosis and hypercalcemia
 - (i) hyperglycemia and hyperlipidemia
 - (j) muscle atrophy
 - (k) arterosclerosis
 - (l) steroid diabetes
- 20 7. An antibody, especially a monoclonal antibody, characterised by specifically binding Cyp7b enzymes.
8. The use of an antibody as claimed in Claim 5 in a test kit for assaying for the presence of Cyp7b enzymes.
9. The use of Cyp7b coding sequences or antisense sequences in the manufacture of
 - 25 a targeted drug for gene therapy of Cyp deficiencies or excesses or for promoting immunogenic processes.

10. The use claimed in Claim 9 wherein a vector is co-administered with a vaccine formulation, whereby on administration, a Cyp7b sequence is expressed and the produced expression product augments an immunogenic property of the vaccine.

11. A process of producing a 7α -hydroxy-substituted steroid which comprises 5 subjecting a corresponding steroid substrate having no substituent in the 7-position to hydroxylation in the presence of a Cyp7b steroid hydroxylase enzyme.

12. A process according to Claim 11 wherein the enzyme is a mouse, rat or human Cyp7b steroid hydroxylase enzyme.

13. A process according to Claim 11 wherein the Cyp7b steroid hydroxylase enzyme 10 has a sequence encoded by DNA coding sequences of Cyp7b enzymes selected from

(a) Coding sequences of DNA molecules comprising the coding sequence for rat Cyp7b set forth in SEQ Id No: 1,

(b) Coding sequences of DNA molecules comprising the coding sequence for mouse Cyp7b set forth in SEQ Id No: 2,

15 (c) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (a) or (b) under standard hybridization conditions defined as 2 x SSC at 65°C.

20 (d) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (a), (b) or (c) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.

14. A process according to Claim 11 wherein the Cyp7b steroid hydroxylase enzyme has a sequence encoded by DNA coding sequences of Cyp7b enzymes selected from

(e) DNA coding sequences selected from the following:

(i) the sequence designated "exon 3" in SEQ Id No 3,
(ii) the sequence designated "exon 4" in SEQ Id No 3, and

(f) Cyp7b steroid hydroxylase-encoding DNA molecules capable of
5 hybridizing with the DNA molecules defined in (e) under standard
hybridization conditions defined as 2 x SSC at 65°C.

(g) Cyp7b steroid hydroxylase encoding DNA molecules capable of
10 hybridizing with the DNA molecule defined in (e) or (f) under
reduced stringency hybridization conditions defined as 6 x SSC at
55°C.

(h) Cyp7b steroid hydroxylase-encoding DNA molecules comprising
contiguous pairs of sequences selected from
15 (i) the sequence designated "exon 3" in SEQ Id No 3,
(ii) the sequence designated "exon 4" in SEQ Id No 3, and

(i) Cyp7b steroid hydroxylase-encoding DNA molecules capable of
hybridizing with the DNA molecules defined in (h) under standard
hybridization conditions defined as 2 x SSC at 65°C.

(j) Cyp7b steroid hydroxylase-encoding DNA molecules capable of
20 hybridizing with the DNA molecule defined in (h) or (i) under
reduced stringency hybridization conditions defined as 6 x SSC at
55°C.

(k) Coding sequences of DNA molecules comprising a contiguous
coding sequence consisting of the sequences "exon 3" and "exon
4" in SEQ Id No 3, and

(l) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (k) under standard hybridization conditions defined as 2 x SSC at 65°C.

5 (m) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (k) or (l) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.

15. A process according to Claim 11 wherein the Cyp7b steroid hydroxylase enzyme has a sequence encoded by DNA coding sequences of Cyp7b enzymes selected from the 10 amino acid sequences contained in SEQ ID NO. 4, SEQ ID NO. 5 or SEQ ID NO 6 or a sequence which has at least 50% homology with one or more of the aforementioned sequences, provided that the capacity to catalyse the introduction of a 7 α -hydroxyl group is not eliminated.

16. A process according to Claim 15 wherein the Cyp7b steroid hydroxylase enzyme 15 has a sequence encoded by a DNA coding sequence which has at least 60% homology, and preferably at least 70% homology with one or more of the aforementioned sequences, provided that the capacity to catalyse the introduction of a 7 α -hydroxyl group is not eliminated.

20 17. A process according to Claim 15 wherein the Cyp7b steroid hydroxylase enzyme has a sequence which differs from the amino acid sequences contained in SEQ ID NO. 4, SEQ ID NO. 5 or SEQ ID NO 6 by not more than 20, preferably not more than 10 and most preferably not more than 5 amino acid substitutions, insertions or deletions.

18. A process according to any preceding claim wherein substrate is a steroid 25 possessing a 3 β -hydroxyl group.

19. A process according to any preceding claim wherein the substrate is a steroid possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, with

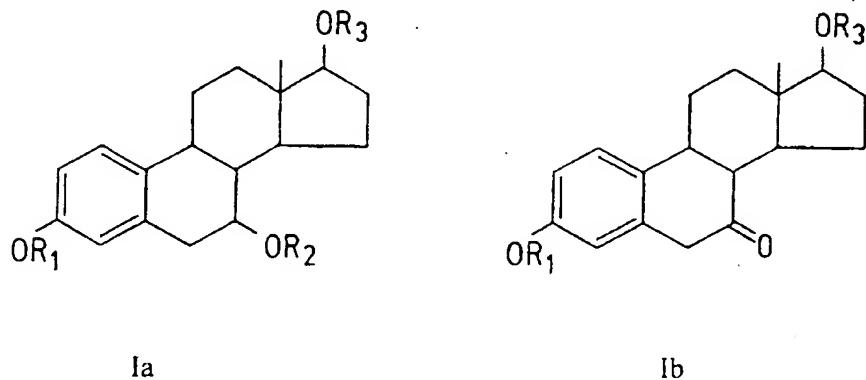
the proviso that where the substrate has the carbon skeleton of cholesterol, the substrate has a hydroxyl group in the 25, 26 or 27-position.

20. A process according to Claim 19 wherein the substrate is 25-hydroxycholesterol, dehydroepiandrosterone, pregnenolone or estradiol.

5 21. A process according to any preceding claim wherein the produced 7α -hydroxy-substituted steroid is 7α -hydroxyestradiol, 7α -hydroxypregnenolone or 7α -hydroxydehydroepiandrosterone.

22. A process according to any preceding claim wherein produced steroid is subjected to an oxidation step to convert an $\text{H}_2\text{O}\text{H}$ to an oxo group.

10 23. A steroid of the formula



wherein OR_1 , OR_2 and OR_3 each independently represents a free hydroxy group, an ether group or an esterified hydroxy group.

24. A steroid according to Claim 23 wherein

15 each of R_1 , R_2 and R_3 may be selected from substituted or unsubstituted C_{1-6} alkyl groups, any such substituents being selected from OH , halogen (F, Cl, Br, I) amino, (C_{1-6}) alkylamino, C_{1-6} dialkylamino, COOH or COOR_4 wherein R_4 represents a C_{1-6} alkyl group which may be unsubstituted or substituted by one of the substituents referred to above; or

OR₁, OR₂ and OR₃ each independently represents an esterified hydroxy group, of the formula R₅COO-, wherein R₅ may be selected from substituted or unsubstituted C₁₋₆ alkyl groups, any such substituents being selected from OH, halogen (F, Cl, Br, I) amino, C₁₋₆ alkylamino, C₁₋₆ dialkylamino, COOH or COOR₄ wherein R₄ represents a C₁₋₆ alkyl group;

5 or

OR₁, OR₂ and OR₃ each independently represents an esterified hydroxy group of formula -OP(OH)₃,
or a pharmacologically acceptable salt of such a compound.

10 25. 7 α -Hydroxyestradiol or 7-oxoestradiol.

26. A steroid as claimed in Claim 23 characterised in that it is a 3 β -hydroxy steroid.

27. A process of producing an oxo-substituted steroid which comprises subjecting 7 α -hydroxyestradiol, 7 α -hydroxypregnенolone or 7 α -hydroxydehydroepiandrosterone to oxidation.

15 28. A method for treating a human or animal requiring therapy for a neuropsychiatric, immune and endocrine disorder or for inducing cognitive enhancement comprising the administration of an effective amount of a 7 α -hydroxy or 7-oxo substituted 3 β -hydroxy-steroid or derivative thereof independently substituted at one or both of the 7-and 3-positions by an ester or ether group.

20 29. A method according to Claim 28 wherein said disorders are selected from

- (a) deficits of cognition in aging
- (b) Alzheimer's disease
- (c) deficits of immune system in aging
- (d) deficits of immune function in HIV infection
- (e) glucocorticoid or mineralocorticoid excess
- (f) diabetes
- (g) depression

- (h) osteoporosis and hypercalcemia
- (I) hyperglycemia and hyperlipidemia
- (j) muscle atrophy
- (k) arterosclerosis
- 5 (l) steroid diabetes

30. A method as claimed in claim 28 wherein the steroid possesses the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol and has a 3β -substituent- OR_1 and/or a 7α -substituent - OR_2 where - OR_1 and - OR_2 each independently represents a free hydroxy, 10 ester or ether group,

wherein each of R_1 and R_2 are independently selected from the group consisting of hydrogen, substituted or unsubstituted C_{1-6} alkyl groups, groups R_5CO- , wherein R_5 may be selected from substituted or unsubstituted C_{1-6} alkyl groups, and groups of the formula - $OP(OH)_3$, wherein any substituents are selected from OH, halogen (F, Cl, Br, I) 15 amino, C_{1-6} alkylamino, C_{1-6} dialkylamino, COOH or $COOR_4$ wherein R_4 represents a C_{1-6} alkyl group; and wherein the compounds may be in free form or in the form of acid addition salts with pharmacologically acceptable anions.

31. A 7α -hydroxy or 7-oxo substituted 3β -hydroxy-steroid possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, or a derivative thereof 20 substituted independently at one or both of the 7- and 3- positions with an ester or ether group for use in therapy.

32. A steroid as claimed in claim 31 selected from 7α -hydroxydehydroepiandrosterone, 7α -hydroxypregnenolone and 7α -hydroxyestradiol.

25 33. A pharmaceutical composition characterised in that it comprises a 7α -hydroxy or 7-oxo substituted 3β -hydroxy steroid possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, or a derivative thereof substituted independently at one or both of the 7- and 3- positions with an ester or ether group, in association with a pharmaceutically acceptable carrier or diluent in a sterile and pyrogen free form.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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5 (A) NAME: British Technology Group Ltd
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(E) COUNTRY: GB
(F) POSTAL CODE (ZIP): EH4 2XU

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40 (A) NAME: Caroline McKenzie LECKIE
(B) STREET: Molecular Medicine Centre, The University of Edinburgh
(C) CITY: Edinburgh
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(F) POSTAL CODE (ZIP): EH4 2XU

45 (ii) TITLE OF INVENTION: NEUROSTEROIDS

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:

5 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: *****

10 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1763 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 1..1245

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCC TTG GAG TAC CAG TAT GTA ATG AAA AAC CCA AAA CAA TTA AGC TTT
 48 Ala Leu Glu Tyr Gln Tyr Val Met Lys Asn Pro Lys Gln Leu Ser Phe
 1 5 10 15

25 GAG AAG TTC AGC CGA AGA TTA TCA GCG AAA GCC TTC TCT GTC AAG AAG
 96 Glu Lys Phe Ser Arg Arg Leu Ser Ala Lys Ala Phe Ser Val Lys Lys
 20 25 30

30 CTG CTA ACT AAT GAC GAC CTT AGC AAT GAC ATT CAC AGA GGC TAT CTT 144
 Leu Leu Thr Asn Asp Asp Leu Ser Asn Asp Ile His Arg Gly Tyr Leu
 35 40 45

CTT TTA CAA GGC AAA TCT CTG GAT GGT CTT CTG GAA ACC ATG ATC CAA 192
 Leu Leu Gln Gly Lys Ser Leu Asp Gly Leu Leu Glu Thr Met Ile Gln
 50 55 60

35 GAA GTA AAA GAA ATA TTT GAG TCC AGA CTG CTA AAA CTC ACA GAT TGG 240
 Glu Val Lys Glu Ile Phe Glu Ser Arg Leu Leu Lys Leu Thr Asp Trp
 65 70 75 80

40 AAT ACA GCA AGA GTA TTT GAT TTC TGT AGT TCA CTG GTA TTT GAA ATC 288
 Asn Thr Ala Arg Val Phe Asp Phe Cys Ser Ser Leu Val Phe Glu Ile
 85 90 95

ACA TTT ACA ACT ATA TAT GGA AAA ATT CTT GCT GCT AAC AAA AAA CAA 336
 Thr Phe Thr Thr Ile Tyr Gly Lys Ile Leu Ala Ala Asn Lys Gln

	100	105	110	
				384
	ATT ATC AGT GAG CTG AGG GAT GAT TTT TTA AAA TTT GAT GAC CAT TTC Ile Ile Ser Glu Leu Arg Asp Asp Phe Leu Lys Phe Asp Asp His Phe			
	115	120	125	
5	CCA TAC TTA GTA TCT GAC ATA CCT ATT CAG CTT CTA AGA AAT GCA GAA Pro Tyr Leu Val Ser Asp Ile Pro Ile Gln Leu Arg Asn Ala Glu			432
	130	135	140	
	TTT ATG CAG AAG AAA ATT ATA AAA TGT CTC ACA CCA GAA AAA GTA GCT Phe Met Gln Lys Lys Ile Ile Lys Cys Leu Thr Pro Glu Lys Val Ala			480
10	145	150	155	160
	CAG ATG CAA AGA CGG TCA GAA ATT GTT CAG GAG AGG CAG GAG ATG CTG Gln Met Gln Arg Arg Ser Glu Ile Val Gln Glu Arg Gln Glu Met Leu			528
	165	170	175	
	AAA AAA TAC TAC GGG CAT GAA GAG TTT GAA ATA GGA GCA CAT CAT CTT Lys Lys Tyr Tyr Gly His Glu Glu Phe Glu Ile Gly Ala His His Leu			576
15	180	185	190	
	GAC GAA ATT GAC AGC TTC CTG CAG TCA ACA GGT CAA AAG AAA GGA CCT Asp Glu Ile Asp Ser Phe Leu Gln Ser Thr Gly Gln Lys Lys Gly Pro			624
	195	200	205	
20	GCA ATG TAT TAT CTT CTT CAG CAT CCA GAA GCT ATG GAA GTC CTG CGT Ala Met Tyr Tyr Leu Leu Gln His Pro Glu Ala Met Glu Val Leu Arg			672
	210	215	220	
	GAC GAA ATT GAC AGC TTC CTG CAG TCA ACA GGT CAA AAG AAA GGA CCT Asp Glu Ile Asp Ser Phe Leu Gln Ser Thr Gly Gln Lys Lys Gly Pro			720
25	225	230	235	240
	GGA ATT TCT GTC CAC TTC ACC AGA GAA CAA TTG GAC AGC TTG GTC TGC Gly Ile Ser Val His Phe Thr Arg Glu Gln Leu Asp Ser Leu Val Cys			768
	245	250	255	
	CTG GAA AGC GCT ATT CTT GAG GTT CTG AGG TTG TGC TCC TAC TCC AGC Leu Glu Ser Ala Ile Leu Glu Val Leu Arg Leu Cys Ser Tyr Ser Ser			816
30	260	265	270	
	ATC ATC CGT GAA GTG CAA GAG GAT ATG GAT TTC AGC TCA GAG AGT AGG Ile Ile Arg Glu Val Gln Glu Asp Met Asp Phe Ser Ser Glu Ser Arg			864
	275	280	285	
35	AGC TAC CGT CTG CGG AAA GGA GAC TTT GTA GCT GTC TTT CCT CCA ATG Ser Tyr Arg Leu Arg Lys Gly Asp Phe Val Ala Val Phe Pro Pro Met			912
	290	295	300	
	ATA CAC AAT GAC CCA GAA GTC TTC GAT GCT CCA AAG GAC TTT AGG TTT Ile His Asn Asp Pro Glu Val Phe Asp Ala Pro Lys Asp Phe Arg Phe			960
40	305	310	315	320
	GAT CGC TTC GTA GAA GAT GGT AAG AAG AAA ACA ACG TTT TTC AAA GGA Asp Arg Phe Val Glu Asp Gly Lys Lys Thr Thr Phe Phe Lys Gly			1008

	325	330	335	
				1056
	GGA AAA AAG CTG AAG AGT TAC ATT ATA CCA TTT GGA CTT GGA ACA AGC Gly Lys Lys Leu Lys Ser Tyr Ile Ile Pro Phe Gly Leu Gly Thr Ser			
	340	345	350	
5	AAA TGT CCA GGC AGA TAC TTT GCA ATT AAT GAA ATG AAG CTA CTA GTG Lys Cys Pro Gly Arg Tyr Phe Ala Ile Asn Glu Met Lys Leu Leu Val			1104
	355	360	365	
10	ATT ATA CTT TTA ACT TAT TTT GAT TTA GAA GTC ATT GAC ACT AAG CCT Ile Ile Leu Leu Thr Tyr Phe Asp Leu Glu Val Ile Asp Thr Lys Pro			1152
	370	375	380	
	ATA GGA CTA AAC CAC AGT CGC ATG TTT CTG GGC ATT CAG CAT CCA GAC Ile Gly Leu Asn His Ser Arg Met Phe Leu Gly Ile Gln His Pro Asp			1200
	385	390	395	400
15	TCT GAC ATC TCA TTT AGG TAC AAG GCA AAA TCT TGG AGA TCC TGA Ser Asp Ile Ser Phe Arg Tyr Lys Ala Lys Ser Trp Arg Ser *			1245
	405	410	415	
	AAGGGTGGCA GAGAAGCTTA GCGGAATAAG GCTGCACATG CTGAGCTCTG TGATTTGCTG			1305
	TAATCCCCAA ATGCAGCCAC TATTCTTGTGTT TGTTAGAAAA TGGCAAATTT TTATTTGATT			1365
	GCGATCCATC CAGTTGTTT TGGGTACCAA AACCTGTCAAT AAAATAAAGC GCTGTCAATGG			1425
20	TGTAAAAAAA TGTATGGCA ATCATTTCAG GATAAGGTA AATAACGTTT TCAAGTTTGT ACTTACTATG ATTTTATCA TTTGTAGTGA ATGTGCTTTT CCAGTAATAA ATTTGCGCCA			1485
	GGGTGATTTT TTTAATTAC TGAAATCCTC TAATATCGGT TTTATGTGCT GCCAGAAAAG			1545
	TGTGCCATCA ATGGACAGTA TAACAATTTC CAGTTTCCA GAGAAGGGAG AAATTAAGCC			1605
	CCATGAGTTA CGCTGTATAA AATTGTTCTC TTCAACTATA ATATCAATAA TGTCTATATC			1665
25	ACCAGGTTAC CTTTGCATTA AATCGAGTTT TGCAAAAG			1725
				1763

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1880 base pairs
 30 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
 35 (B) LOCATION: 81..1604

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

60 GGCAGGCACA GCCTCTGGTC TAAGAAGAGA GGGCACTGTG CAGAAGCCAT CGCTCCCTAC
 110 AGAGCCGCCA GCTCGTCGGG ATG CAG GGA GCC ACG ACC CTA GAT GCC GCC
 Met Gln Gly Ala Thr Thr Leu Asp Ala Ala
 5 420 425
 158 TCG CCA GGG CCT CTC GCC CTC CTA GGC CTT CTC TTT GCC GCC ACC TTA
 Ser Pro Gly Pro Leu Ala Leu Leu Gly Leu Leu Phe Ala Ala Thr Leu
 430 435 440
 206 CTG CTC TCG GCC CTG TTC CTC CTC ACC CGG CGC ACC AGG CGC CCT CGT
 10 Leu Leu Ser Ala Leu Phe Leu Leu Thr Arg Arg Thr Arg Arg Pro Arg
 445 450 455
 254 GAA CCA CCC TTG ATA AAA GGT TGG CTT CCT TAT CTT GGC ATG GCC CTG
 Glu Pro Pro Leu Ile Lys Gly Trp Leu Pro Tyr Leu Gly Met Ala Leu
 460 465 470
 302 15 AAA TTC TTT AAG GAT CCG TTA ACT TTC TTG AAA ACT CTT CAA AGG CAA
 Lys Phe Phe Lys Asp Pro Leu Thr Phe Leu Lys Thr Leu Gln Arg Gln
 475 480 485
 350 20 CAT GGT GAC ACT TTC ACT GTC TTC CTT GTG GGG AAG TAT ATA ACA TTT
 His Gly Asp Thr Phe Thr Val Phe Leu Val Gly Lys Tyr Ile Thr Phe
 490 495 500 505
 398 25 GTT CTG AAC CCT TTC CAG TAC CAG TAT GTA ACG AAA AAC CCA AAA CAA
 Val Leu Asn Pro Phe Gln Tyr Gln Tyr Val Thr Lys Asn Pro Lys Gln
 510 515 520
 446 30 TTA AGC TTT CAG AAG TTC AGC AGC CGA TTA TCA GCG AAA GCC TTC TCT
 Leu Ser Phe Gln Lys Phe Ser Ser Arg Leu Ser Ala Lys Ala Phe Ser
 525 530 535
 494 35 GTA AAG AAG CTG CTT ACT GAT GAC GAC CTT AAT GAA GAC GTT CAC AGA
 Val Lys Leu Leu Thr Asp Asp Asp Leu Asn Glu Asp Val His Arg
 540 545 550
 542 40 GCC TAT CTA CTT CTA CAA GGC AAA CCT TTG GAT GCT CTT CTG GAA ACT
 Ala Tyr Leu Leu Gln Gly Lys Pro Leu Asp Ala Leu Leu Glu Thr
 555 560 565
 590 35 ATG ATC CAA GAA GTA AAA GAA TTA TTT GAG TCC CAA CTG CTA AAA ATC
 Met Ile Gln Glu Val Lys Glu Leu Phe Glu Ser Gln Leu Leu Lys Ile
 570 575 580 585
 638 45 ACA GAT TGG AAC ACA GAA AGA ATA TTT GCA TTC TGT GGC TCA CTG GTA
 Thr Asp Trp Asn Thr Glu Arg Ile Phe Ala Phe Cys Gly Ser Leu Val
 590 595 600
 686 40 TTT GAG ATC ACA TTT GCG ACT CTA TAT GGA AAA ATT CTT GCT GGT AAC
 Phe Glu Ile Thr Phe Ala Thr Leu Tyr Gly Lys Ile Leu Ala Gly Asn
 605 610 615
 734 45 AAG AAA CAA ATT ATC AGT GAG CTA AGG GAT GAT TTT TTT AAA TTT GAT

	Lys Lys Gln Ile Ile Ser Glu Leu Arg Asp Asp Phe Phe Lys Phe Asp			
	620	625	630	
	GAC ATG TTC CCA TAC TTA GTA TCT GAC ATA CCT ATT CAG CTT CTA AGA			782
5	Asp Met Phe Pro Tyr Leu Val Ser Asp Ile Pro Ile Gln Leu Leu Arg	635	640	645
	AAT GAA GAA TCT ATG CAG AAG AAA ATT ATA AAA TGC CTC ACA TCA GAA			830
	Asn Glu Glu Ser Met Gln Lys Lys Ile Ile Lys Cys Leu Thr Ser Glu	650	655	660
	665			
10	AAA GTA GCT CAG ATG CAA GGA CAG TCA AAA ATT GTT CAG GAA AGC CAA			878
	Lys Val Ala Gln Met Gln Gly Gln Ser Lys Ile Val Gln Glu Ser Gln	670	675	680
	685			
	GAT CTG CTG AAA AGA TAC TAT AGG CAT GAC GAT TCT GAA ATA GGA GCA			926
	Asp Leu Leu Lys Arg Tyr Tyr Arg His Asp Asp Ser Glu Ile Gly Ala	685	690	695
15	CAT CAT CTT GGC TTT CTC TGG GCC TCT CTA GCA AAC ACC ATT CCA GCT			974
	His His Leu Gly Phe Leu Trp Ala Ser Leu Ala Asn Thr Ile Pro Ala	700	705	710
	715			
20	ATG TTC TGG GCA ATG TAT TAT ATT CTT CGG CAT CCT GAA GCT ATG GAA			1022
	Met Phe Trp Ala Met Tyr Tyr Ile Leu Arg His Pro Glu Ala Met Glu	720	725	
	730			
	GCC CTG CGT GAC GAA ATT GAC AGT TTC CTG CAG TCA ACA GGT CAA AAG			1070
	Ala Leu Arg Asp Glu Ile Asp Ser Phe Leu Gln Ser Thr Gly Gln Lys	735	740	745
25	AAA GGG CCT GGA ATT TCA GTC CAC TTC ACC AGA GAA CAA TTG GAC AGC			1118
	Lys Gly Pro Gly Ile Ser Val His Phe Thr Arg Glu Gln Leu Asp Ser	750	755	760
	765			
	TTG GTC TGC CTG GAA AGC ACT ATT CTT GAG GTT CTG AGG CTG TGC TCA			1166
	Leu Val Cys Leu Glu Ser Thr Ile Leu Glu Val Leu Arg Leu Cys Ser	770	775	
30	TAC TCC AGC ATC ATC CGA GAA GTG CAG GAG GAT ATG AAT CTC AGC TTA			1214
	Tyr Ser Ser Ile Ile Arg Glu Val Gln Glu Asp Met Asn Leu Ser Leu	780	785	790
	795			
	GAG AGT AAG AGT TTC TCT CTG CGG AAA GGA GAT TTT GTA GCC CTC TTT			1262
35	Glu Ser Lys Ser Phe Ser Leu Arg Lys Gly Asp Phe Val Ala Leu Phe	800	805	
	810			
	CCT CCA CTC ATA CAC AAT GAC CCG GAA ATC TTC GAT GCT CCA AAG GAA			1310
	Pro Pro Leu Ile His Asn Asp Pro Glu Ile Phe Asp Ala Pro Lys Glu	815	820	825
40	TTT AGG TTC GAT CGG TTC ATA GAA GAT GGT AAG AAG AAA AGC ACG TTT			1358
	Phe Arg Phe Asp Arg Phe Ile Glu Asp Gly Lys Lys Ser Thr Phe	830	835	840
	830			
	TTC AAA GGA GGG AAG AGG CTG AAG ACT TAC GTT ATG CCT TTT GGA CTC			1406
	Phe Lys Gly Lys Arg Leu Lys Thr Tyr Val Met Pro Phe Gly Leu			

	845	850	855	
	GGA ACA AGC AAA TGT CCA GGG AGA TAT TTT GCA GTG AAC GAA ATG AAG Gly Thr Ser Lys Cys Pro Gly Arg Tyr Phe Ala Val Asn Glu Met Lys 860	865	870	1454
5	CTA CTG CTG ATT GAG CTT TTA ACT TAT TTT GAT TTA GAA ATT ATC GAC Leu Leu Leu Ile Glu Leu Leu Thr Tyr Phe Asp Leu Glu Ile Ile Asp 875	880	885	1502
10	AGG AAG CCT ATA GGG CTA AAT CAC AGT CGG ATG TTT TTA GGT ATT CAG Arg Lys Pro Ile Gly Leu Asn His Ser Arg Met Phe Leu Gly Ile Gln 890	895	900	1550
	CAC CCC GAT TCT GCC GTC TCC TTT AGG TAC AAA GCA AAA TCT TGG AGA His Pro Asp Ser Ala Val Ser Phe Arg Tyr Lys Ala Lys Ser Trp Arg 910	915	920	1598
15	AGC TGA AAGTGTGGCA GAGAAGCTTT GCAGAGTAAG GCTGCATGTG CTGAGCTCCG Ser *			1654
	TGATTTGGTG CACTCCCCCA AATGCAACCG CTACTCTTGT TTGAAAATGG CAAATTTATA			1714
	TTTGGTTGAG ATCAATCCAG TTGGTTTG GTCACAAAAC CTGTCATAAA ATAAAGCAGT			1774
	GTGATGGTTT AAAAAATGTC ATGGCAATCA TTTCAGGATA AGGTAAAATA ACATTTCAA			1834
20	GTTTGTACTT ACTATGATTT TTATCATTG TAGTGAATGT GCTTTT			1880
	(2) INFORMATION FOR SEQ ID NO: 3:			
	(i) SEQUENCE CHARACTERISTICS:			
25	(A) LENGTH: 3846 base pairs			
	(B) TYPE: nucleic acid			
	(C) STRANDEDNESS: double			
	(D) TOPOLOGY: linear			
	(ii) MOLECULE TYPE: DNA (genomic)			
	(ix) FEATURE:			
30	(A) NAME/KEY: CDS			
	(B) LOCATION: 831..2078			
	(ix) FEATURE:			
	(A) NAME/KEY: exon (3)			
	(B) LOCATION: 831..1422			
35	(ix) FEATURE:			
	(A) NAME/KEY: intron			
	(B) LOCATION: 1423..1872			

(ix) FEATURE:

- (A) NAME/KEY: exon (4)
- (B) LOCATION: 1873..2078

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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	ATGGATGAAT GGATGTGTT TAGTTGGAA TCCTGTGTCA GTCACAGTC AATATGTGAC	120
	CTTGAACATG TTATTAATC TCCCACATCC ATAAAAGTGA AAATGCTGGC ATTAGTGGAT	180
	TTTGCCAGT GTTGAATTAG ACATTTATT GTGAGTACCT GCTCCATACA GTATGGTCAT	240
10	TTATTTGAGT TAAAATTGTT GTATTTGAAAC AAAACTCAGA TGACACCTAA GCATGAAAAA GCTCTTTATG AAGTATAAAAT ACTCAGAAAT GGAATGGCAT GTGCCAATT TGTGTTCTGC	300
	TTTATTGAGG GAAATATATG AGAAGTATT AAGTCAGGGG ATTATGAGGA ATATTTAAAG	360
	GATANNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	420
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	480
15	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNTCTAGA GTGTTTCCA CCATTTCA	540
	AAGGAAACAT GTAGTGTACC TTCGAATGAA ATGGATTGT ATTAAACTTT TTGCCTTAGT	600
	TATTAGGGTC TTTCTAATT TTGATTAACA TATTTTTTA ATTGTGGTG TTTATTTCTG	660
	TTTTTATTAA CAAACGAAC CATATGCTCC TCTCTTTT TTTTTTTCT GGAAAGTACA	720
20	TAACATTTAT ACCTGGACCC TTCCAGTACC AGCTAGTGAT AAAAAATCAT AAACAATTAA GCTTTCGAGT ATCTTCTAAT AAATTATCAG AGAAAGCATT TAGCATAGT CAGTTGCAA	780
	AAAATCATGA CATGAATGAT GAGCTTCACC TCTGCTATCA ATTTTGCAA GGAAATCTT	840
	TGGACATACT CTTGGAAAGC ATGATGCAGA ATCTAAAACA AGTTTTGAA CCCCAGCTGT	900
	TAAAAACAC AAGTTGGGAC ACGGCAGAAC TGTATCCATT CTGCAGCTCA ATAATATTG	960
25	AGATCACATT TACAACATA TATGGAAAAG TTATTGTTG TGACACAAAC AAATTTATTA GTGAGCTAAG AGATGATT TTAAAATTG ATGACAAGTT TGCAATTGAA GTATCCAACA	1020
	TACCCATTGA GCTTCTAGGA AATGTCAAGT CTATTAGAGA GAAAATTATA AAATGCTCT	1080
	CATCAGAAAA GTTAGCCAAG ATGCAAGGAT GGTCAGAAGT TTTTCAAAGC AGGCAAGATG	1140
	ACCTGGAGAA ATATTATGTG CACGAGGACC TTGAAATAGG AGGTAAGAAC TTCTGAATGA	1200
30	GCACCTGCCT AAATAAAAT CATTACATA GACCTCTGAA ATAAAAAAAG ACAAAATGGC	1260
		1320
		1380
		1440
		1500

7	GACCTTGAAA ATTTTTTAT GCTCTTCTA ATTGGCTAAT GATAAATGTT TACTCTGATA	1560
	TAACCTCTAT AATTGATATT TTTTTTTTG CTGAGGTGGT AAACAGATAAC TTAATGGTGA	1620
	TAATGAGAAA GCGTATAACT AAGCTGCATT TATCCCTCTT ATCTCATCCC CGACCACACC	1680
5	GCCCCCCCCA TACACATTAC ATTTTAAACT ATTCTCATTA AGCAGAAAAT TAGACTTCAG	1740
	AAGCCTATTG GTTCTCATTA GCATGCAGTG ATCCTGGCT GGTCTGTGTC CTAACATCTT	1800
	TTAATTAGCA CACTGCAAAT CTAATCAGTG TAATAAACGC TATTAATCTT CCTTTACACT	1860
	TATTTCTCC CACACATCAT TTAGGCTTTC TCTGGGCCTC TGTGGCAAAC ACTATTCCAA	1920
	CTATGTTCTG GGCAACGTAT TATCTCTGC GGCACCCAGA AGCTATGGCA GCAGTGCCTG	1980
10	ACGAAATTGA CCGTTGCTG CAGTCAACAG GTCAAAAGGA AGGGTCTGGA TTTCCCATCC	2040
	ACCTCACCAAG AGAACAAATTG GACAGCCTAA TCTGCCTAGG TAATTATTTT ATCTGTTATG	2100
	AAGAAAGAAG GTACCTCTCT GCAAACTCGG TTTATCACTC ATAGCTGTTT ACAAGAGGTA	2160
	GAGGACACAG CTGCTAATTG ACATAATAAC TCCCATTAC ATCAATTATA AATTATGTAG	2220
	TTTATAGCCG TAGATCATCT CATTGCATGT AAACATAAGG CCTATGTAAT TAACTGTGTA	2280
15	ATGTATGTAA AATTCTAACCC AAAGCTNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2340
	NNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2400
	NNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2460
	NNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2520
	NNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2580
20	NNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2640
	NNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2700
	NNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2760
	NNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2820
	NNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNNN	2880
25	AAGTTAAATT CCATACCAAT GAGTTATTCT CTC TGTATTGACA TTTCATCTGC	2940
	GGTATCCTT AGGGTACAAT GAGTTATTCT CTC TGTATTGACA TTTCATCTGC	3000
	GGTATCCTT AGGGTACAAT ATTCCAAGTT TCTTTAGACA AACGCAGGAA CAAATGTTCA	3060
	CATATTCTG TTTCTTATT CCTTGACAA GTAGGCGAGC ATTTAGCCT ATGTTGGTCT	3120
	CAAAAAAAAT CTTTAAATA TGTTCCAGGT TCTTTAATGG GACCTTCAG GAGCAAAAGT	3180

CCTCCCAGGT TTGGTCAATG TTCACCCCTCN GTGGCCATTG AGGAAAATGC CCNNNNNGTT	3240
CTAGAGATTG TTCTCACTTC TCAGGCTAAG GCCCATTGAG CAATGCCAGA AAGCATGCCT	3300
TATACTAGCA GTCAATTGAG AAGTTTGTAG TTTGTGTCTT TAGCATAGGT TATCAAATAA	3360
ATTTTATATT TNCTTTAAA AAAATCTCAA CATTACTAAA ATACAAATAT CCTTTTATT	3420
5 TTCTTGCAG AATTATCGGG GAACAAATCC AGAAAATTG TGAAATTTC GGGTAGTTGC	3480
TCCACTTGAT ACACAGTATT TCTGCATATT GTAATTCTA TGAAGATCTA GGTTGCATT	3540
CCCATAACATT CAAGCAGTTT CCATTGCATT TTTATGAATA AGATGACGCA TACTGGGAAG	3600
TAAGGCAAAT ACACTAAAAG GAATATGTGT TTGTATTCTG TATAGTTATT ACTCTTAAAA	3660
AAAGTAGTTG TAATTCCACTT CTTTCAACTT TTTGCTATTA AAAAATCATT	3720
10 TTTAAATTTC AGTATTAAAG CAGAAACATT TAAATTATT AGACCAGAAA AATAACAGAT	3780
TCTAGAACTA TAATTGAAT CCATTAAAGC CCATAGCTAG AGCTAGAGAT TTTCACTATT	3840
GGATCC	3846

(2) INFORMATION FOR SEQ ID NO: 4:

15	(i) SEQUENCE CHARACTERISTICS:															
	(A) LENGTH: 415 amino acids															
	(B) TYPE: amino acid															
	(D) TOPOLOGY: linear															
	(ii) MOLECULE TYPE: protein															
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:															
20	Ala	Leu	Glu	Tyr	Gln	Tyr	Val	Met	Lys	Asn	Pro	Lys	Gln	Leu	Ser	Phe
	1				5			10						15		
	Glu Lys Phe Ser Arg Arg Leu Ser Ala Lys Ala Phe Ser Val Lys Lys															
		20			25			30								
25	Leu	Leu	Thr	Asn	Asp	Asp	Leu	Ser	Asn	Asp	Ile	His	Arg	Gly	Tyr	Leu
	35				40						45					
	Leu Leu Gln Gly Lys Ser Leu Asp Gly Leu Leu Glu Thr Met Ile Gln															
		50			55			60								
	Glu Val Lys Glu Ile Phe Glu Ser Arg Leu Leu Lys Leu Thr Asp Trp															
		65			70			75			80					
30	Asn	Thr	Ala	Arg	Val	Phe	Asp	Phe	Cys	Ser	Ser	Leu	Val	Phe	Glu	Ile
	85				90							95				
	Thr Phe Thr Thr Ile Tyr Gly Lys Ile Leu Ala Ala Asn Lys Lys Gln															
		100			105			110								
	Ile Ile Ser Glu Leu Arg Asp Asp Phe Leu Lys Phe Asp Asp His Phe															

	115	120	125
	Pro Tyr Leu Val Ser Asp Ile Pro Ile Gln Leu Leu Arg Asn Ala Glu		
	130	135	140
	Phe Met Gln Lys Lys Ile Ile Lys Cys Leu Thr Pro Glu Lys Val Ala		
5	145	150	155
	Gln Met Gln Arg Arg Ser Glu Ile Val Gln Glu Arg Gln Glu Met Leu		
	165	170	175
	Lys Lys Tyr Tyr Gly His Glu Glu Phe Glu Ile Gly Ala His His Leu		
	180	185	190
10	Gly Leu Leu Trp Ala Ser Leu Ala Asn Thr Ile Pro Ala Met Phe Trp		
	195	200	205
	Ala Met Tyr Tyr Leu Leu Gln His Pro Glu Ala Met Glu Val Leu Arg		
	210	215	220
	Asp Glu Ile Asp Ser Phe Leu Gln Ser Thr Gly Gln Lys Lys Gly Pro		
15	225	230	235
	Gly Ile Ser Val His Phe Thr Arg Glu Gln Leu Asp Ser Leu Val Cys		
	245	250	255
	Leu Glu Ser Ala Ile Leu Glu Val Leu Arg Leu Cys Ser Tyr Ser Ser		
	260	265	270
20	Ile Ile Arg Glu Val Gln Glu Asp Met Asp Phe Ser Ser Glu Ser Arg		
	275	280	285
	Ser Tyr Arg Leu Arg Lys Gly Asp Phe Val Ala Val Phe Pro Pro Met		
	290	295	300
	Ile His Asn Asp Pro Glu Val Phe Asp Ala Pro Lys Asp Phe Arg Phe		
25	305	310	315
	Asp Arg Phe Val Glu Asp Gly Lys Lys Lys Thr Thr Phe Phe Lys Gly		
	325	330	335
	Gly Lys Lys Leu Lys Ser Tyr Ile Ile Pro Phe Gly Leu Gly Thr Ser		
	340	345	350
30	Lys Cys Pro Gly Arg Tyr Phe Ala Ile Asn Glu Met Lys Leu Leu Val		
	355	360	365
	Ile Ile Leu Leu Thr Tyr Phe Asp Leu Glu Val Ile Asp Thr Lys Pro		
	370	375	380
	Ile Gly Leu Asn His Ser Arg Met Phe Leu Gly Ile Gln His Pro Asp		
35	385	390	395
	Ser Asp Ile Ser Phe Arg Tyr Lys Ala Lys Ser Trp Arg Ser *		
	405	410	415

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 508 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Gln Gly Ala Thr Thr Leu Asp Ala Ala Ser Pro Gly Pro Leu Ala
 1 5 10 15

10 Leu Leu Gly Leu Leu Phe Ala Ala Thr Leu Leu Leu Ser Ala Leu Phe
 20 25 30

Leu Leu Thr Arg Arg Thr Arg Arg Pro Arg Glu Pro Pro Leu Ile Lys
 35 40 45

15 Gly Trp Leu Pro Tyr Leu Gly Met Ala Leu Lys Phe Phe Lys Asp Pro
 50 55 60

Leu Thr Phe Leu Lys Thr Leu Gln Arg Gln His Gly Asp Thr Phe Thr
 65 70 75 80

Val Phe Leu Val Gly Lys Tyr Ile Thr Phe Val Leu Asn Pro Phe Gln
 85 90 95

20 Tyr Gln Tyr Val Thr Lys Asn Pro Lys Gln Leu Ser Phe Gln Lys Phe
 100 105 110

Ser Ser Arg Leu Ser Ala Lys Ala Phe Ser Val Lys Lys Leu Leu Thr
 115 120 125

25 Asp Asp Asp Leu Asn Glu Asp Val His Arg Ala Tyr Leu Leu Gln
 130 135 140

Gly Lys Pro Leu Asp Ala Leu Leu Glu Thr Met Ile Gln Glu Val Lys
 145 150 155 160

Glu Leu Phe Glu Ser Gln Leu Leu Lys Ile Thr Asp Trp Asn Thr Glu
 165 170 175

30 Arg Ile Phe Ala Phe Cys Gly Ser Leu Val Phe Glu Ile Thr Phe Ala
 180 185 190

Thr Leu Tyr Gly Lys Ile Leu Ala Gly Asn Lys Lys Gln Ile Ile Ser
 195 200 205

35 Glu Leu Arg Asp Asp Phe Phe Lys Phe Asp Asp Met Phe Pro Tyr Leu
 210 215 220

Val Ser Asp Ile Pro Ile Gln Leu Leu Arg Asn Glu Glu Ser Met Gln
 225 230 235 240

Lys Lys Ile Ile Lys Cys Leu Thr Ser Glu Lys Val Ala Gln Met Gln

	245	250	255
	Gly Gln Ser Lys Ile Val Gln Glu Ser Gln Asp Leu Leu Lys Arg Tyr		
	260	265	270
	Tyr Arg His Asp Asp Ser Glu Ile Gly Ala His His Leu Gly Phe Leu		
5	275	280	285
	Trp Ala Ser Leu Ala Asn Thr Ile Pro Ala Met Phe Trp Ala Met Tyr		
	290	295	300
	Tyr Ile Leu Arg His Pro Glu Ala Met Glu Ala Leu Arg Asp Glu Ile		
	305	310	315
10	Asp Ser Phe Leu Gln Ser Thr Gly Gln Lys Lys Gly Pro Gly Ile Ser		
	325	330	335
	Val His Phe Thr Arg Glu Gln Leu Asp Ser Leu Val Cys Leu Glu Ser		
	340	345	350
	Thr Ile Leu Glu Val Leu Arg Leu Cys Ser Tyr Ser Ser Ile Ile Arg		
15	355	360	365
	Glu Val Gln Glu Asp Met Asn Leu Ser Leu Glu Ser Lys Ser Phe Ser		
	370	375	380
	Leu Arg Lys Gly Asp Phe Val Ala Leu Phe Pro Pro Leu Ile His Asn		
	385	390	395
20	Asp Pro Glu Ile Phe Asp Ala Pro Lys Glu Phe Arg Phe Asp Arg Phe		
	405	410	415
	Ile Glu Asp Gly Lys Lys Ser Thr Phe Phe Lys Gly Gly Lys Arg		
	420	425	430
	Leu Lys Thr Tyr Val Met Pro Phe Gly Leu Gly Thr Ser Lys Cys Pro		
25	435	440	445
	Gly Arg Tyr Phe Ala Val Asn Glu Met Lys Leu Leu Ile Glu Leu		
	450	455	460
	Leu Thr Tyr Phe Asp Leu Glu Ile Ile Asp Arg Lys Pro Ile Gly Leu		
	465	470	475
30	Asn His Ser Arg Met Phe Leu Gly Ile Gln His Pro Asp Ser Ala Val		
	485	490	495
	Ser Phe Arg Tyr Lys Ala Lys Ser Trp Arg Ser *		
	500	505	

(2) INFORMATION FOR SEQ ID NO: 6:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 266 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

10 Gly Lys Tyr Ile Thr Phe Ile Pro Gly Pro Phe Gln Tyr Gln Leu Val
 1 5 10 15

Ile Lys Asn His Lys Asn Leu Ser Phe Arg Val Ser Ser Asn Lys Leu
 20 25 30

Ser Glu Lys Ala Phe Ser Ile Ser Gln Leu Gln Lys Asn His Asp Met
 35 40 45

15 Asn Asp Glu Leu His Leu Cys Tyr Gln Phe Leu Gln Gly Lys Ser Leu
 50 55 60

Asp Ile Leu Leu Glu Ser Met Met Gln Asn Leu Lys Gln Val Phe Glu
 65 70 75 80

20 Pro Gln Leu Leu Lys Thr Thr Ser Trp Asp Thr Ala Glu Leu Tyr Pro
 85 90 95

Phe Cys Ser Ser Ile Ile Phe Glu Ile Thr Phe Thr Thr Ile Tyr Gly
 100 105 110

Lys Val Ile Val Cys Asp Asn Asn Lys Phe Ile Ser Glu Leu Arg Asp
 115 120 125

25 Asp Phe Leu Lys Phe Asp Asp Lys Phe Ala Tyr Leu Val Ser Asn Ile
 130 135 140

Pro Ile Glu Leu Leu Gly Asn Val Lys Ser Ile Arg Glu Lys Ile Ile
 145 150 155 160

30 Lys Cys Phe Ser Ser Glu Lys Leu Ala Lys Met Gln Gly Trp Ser Glu
 165 170 175

Val Phe Gln Ser Arg Gln Asp Asp Leu Glu Lys Tyr Tyr Val His Glu
 180 185 190

Asp Leu Glu Ile Gly Ala His His Phe Gly Phe Leu Trp Val Ser Val
 195 200 205

35 Ala Ser Thr Ile Pro Thr Met Phe Trp Ala Thr Tyr Tyr Leu Leu Arg
 210 215 220

His Pro Glu Ala Met Ala Ala Val Arg Asp Glu Ile Asp Arg Leu Leu
225 230 235 240

Gln Ser Thr Gly Gln Lys Glu Gly Ser Gly Phe Pro Ile His Leu Thr
245 250 255

5 Arg Glu Gln Leu Asp Ser Leu Ile Cys Leu
260 265

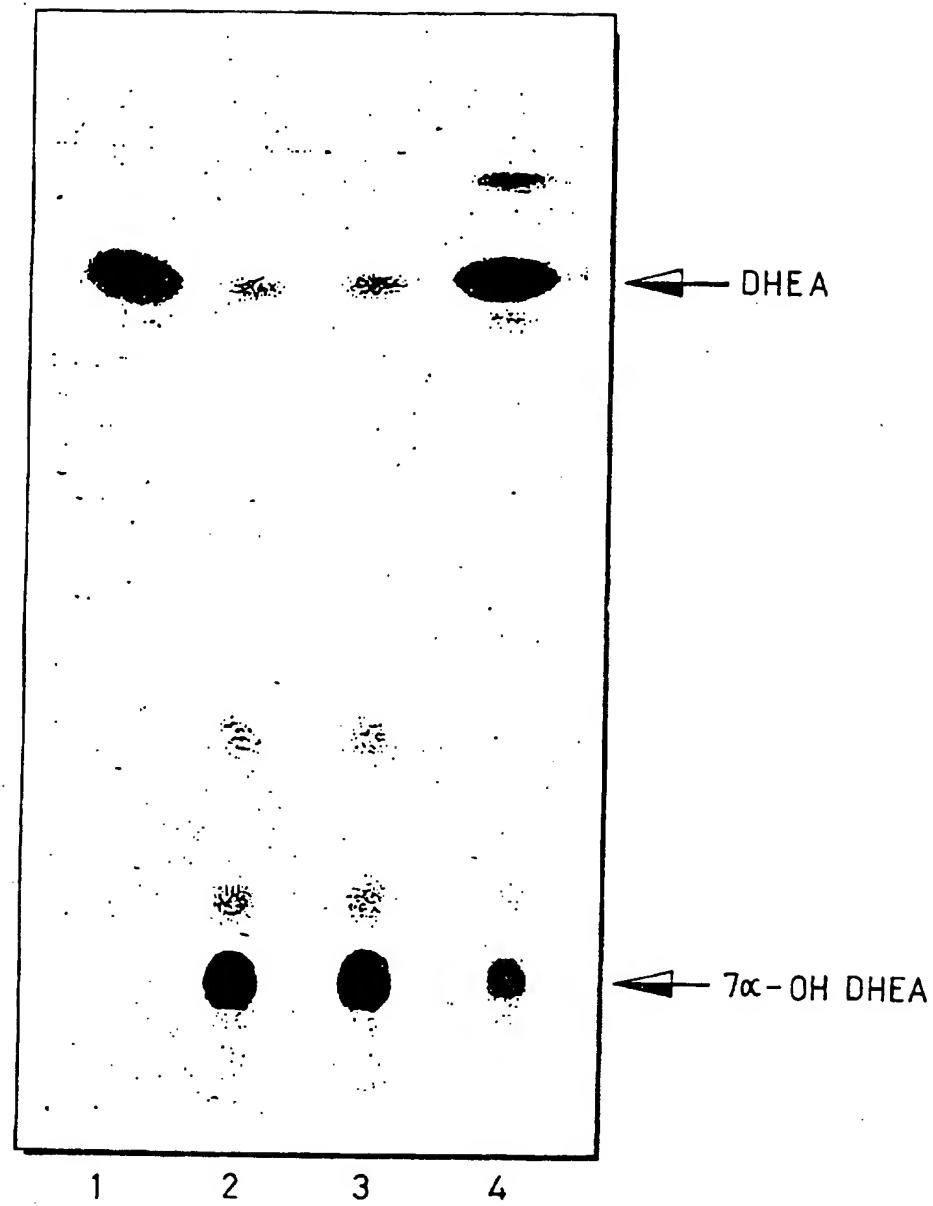


Fig. 1

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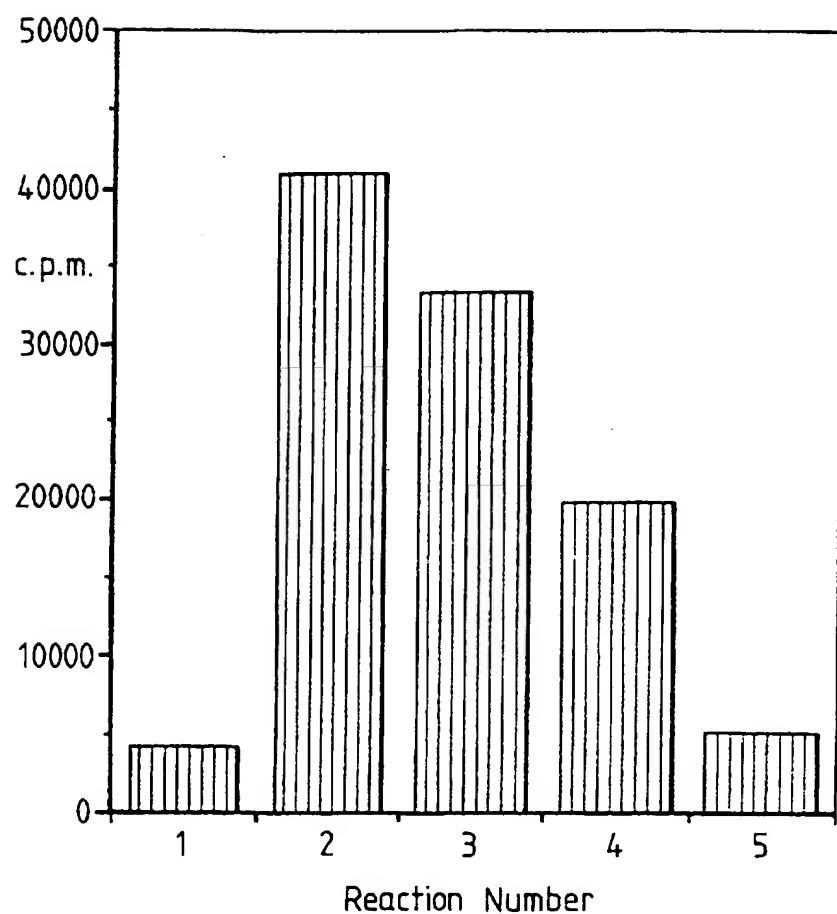
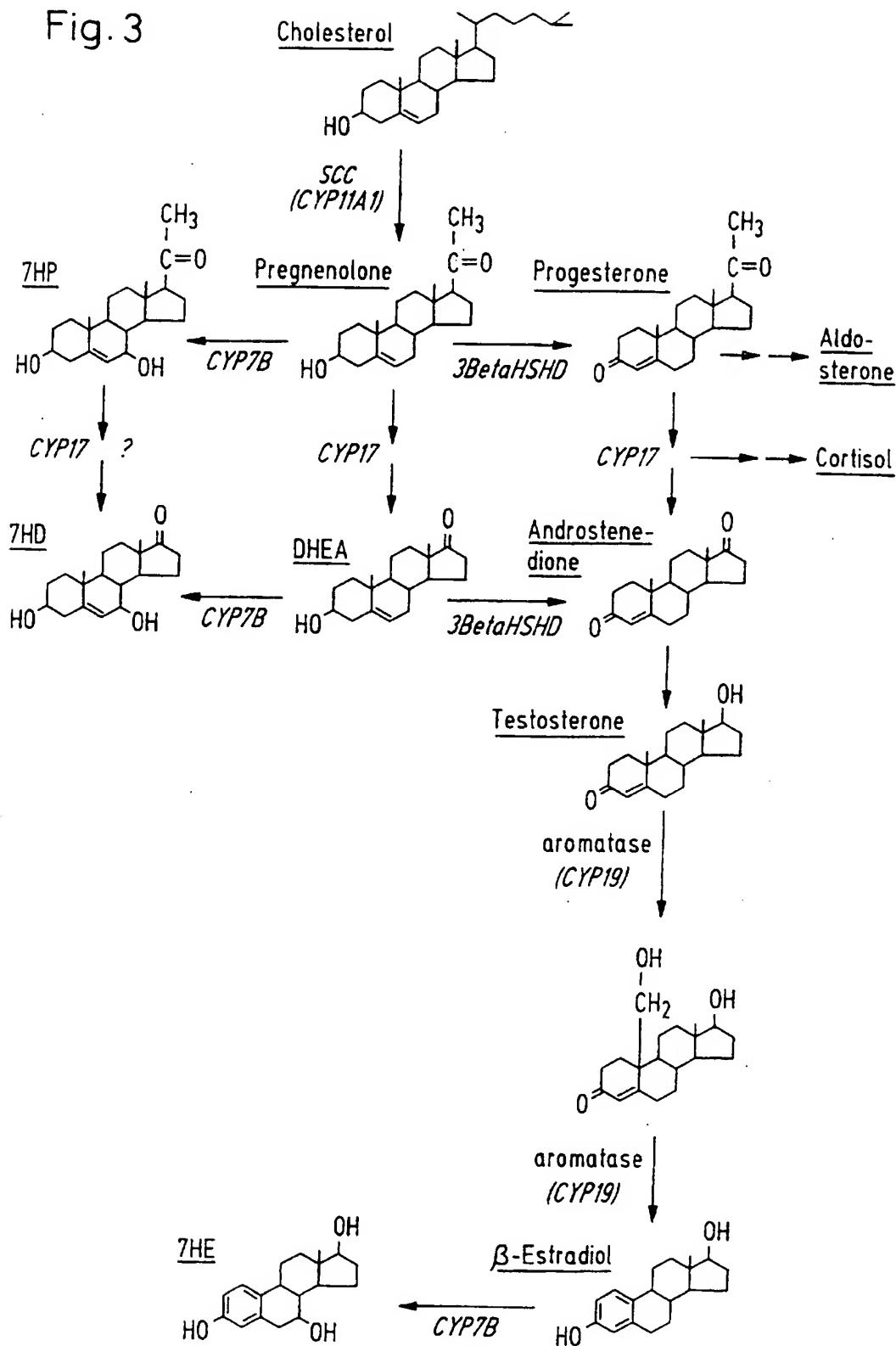


Fig. 2

Fig. 3



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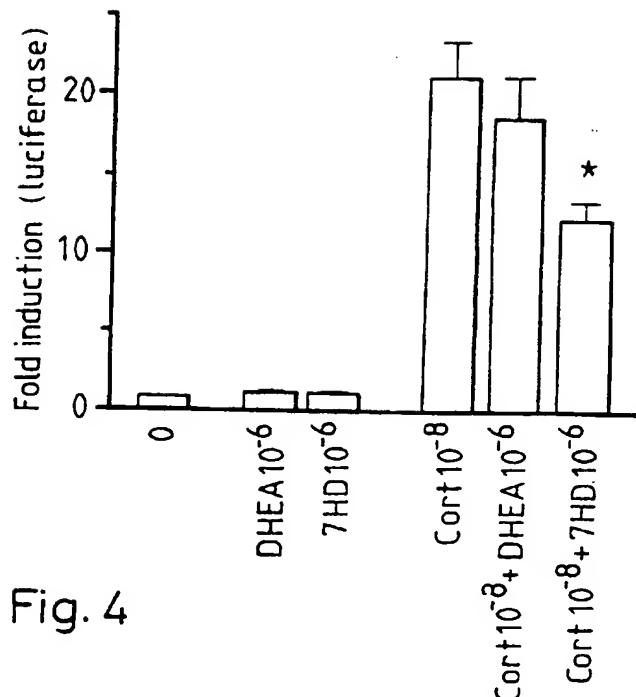


Fig. 4

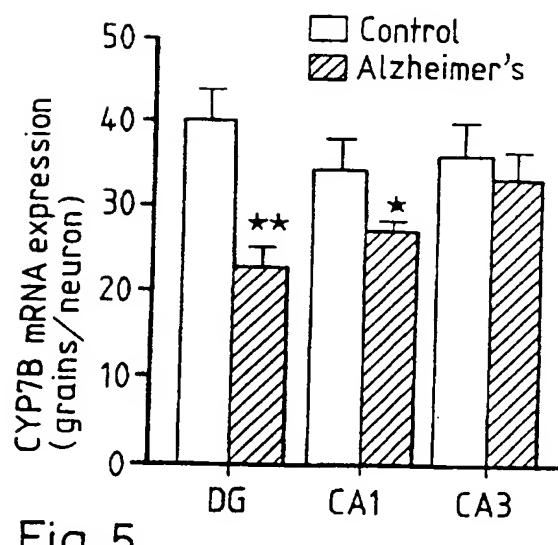


Fig. 5

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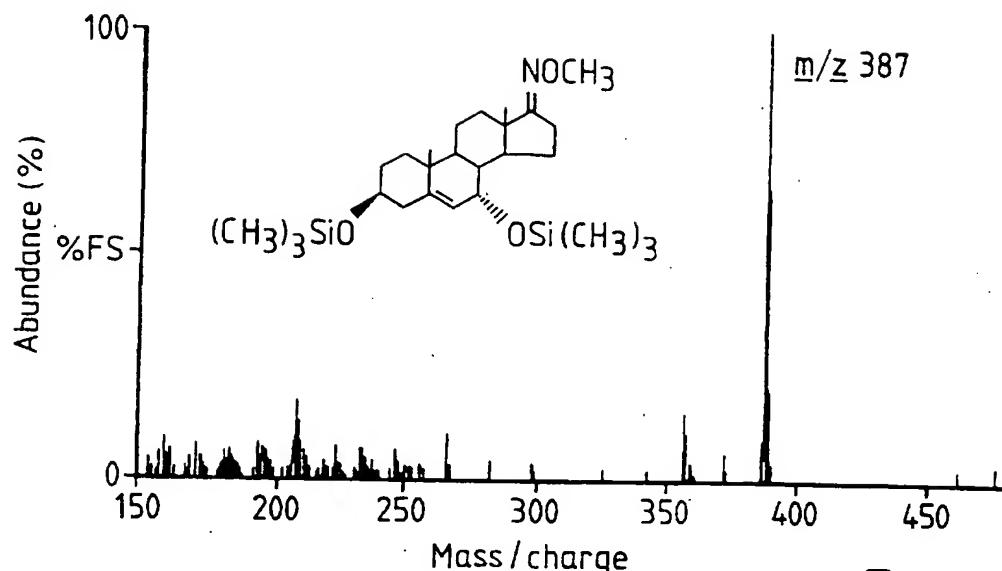


Fig. 6a

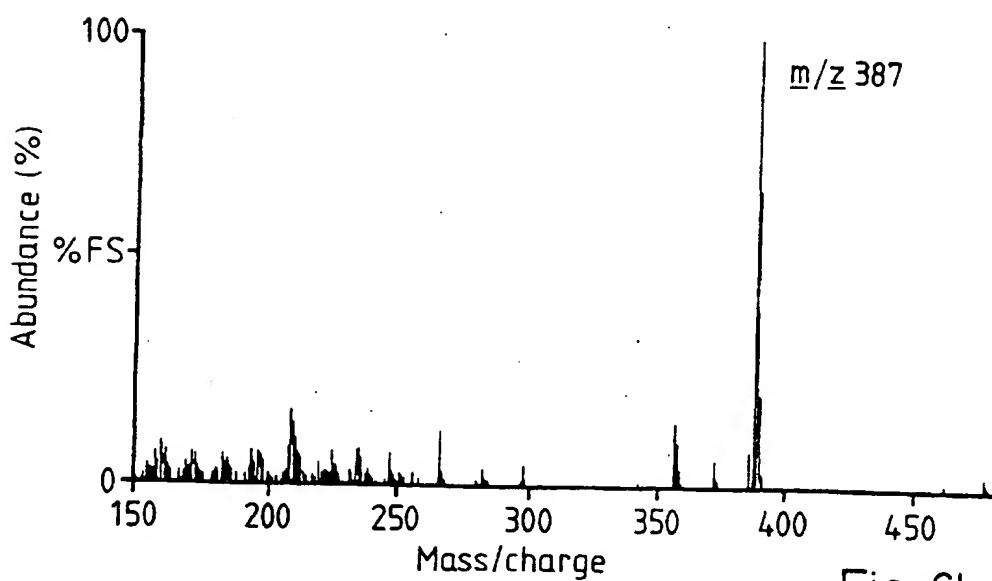


Fig. 6b

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